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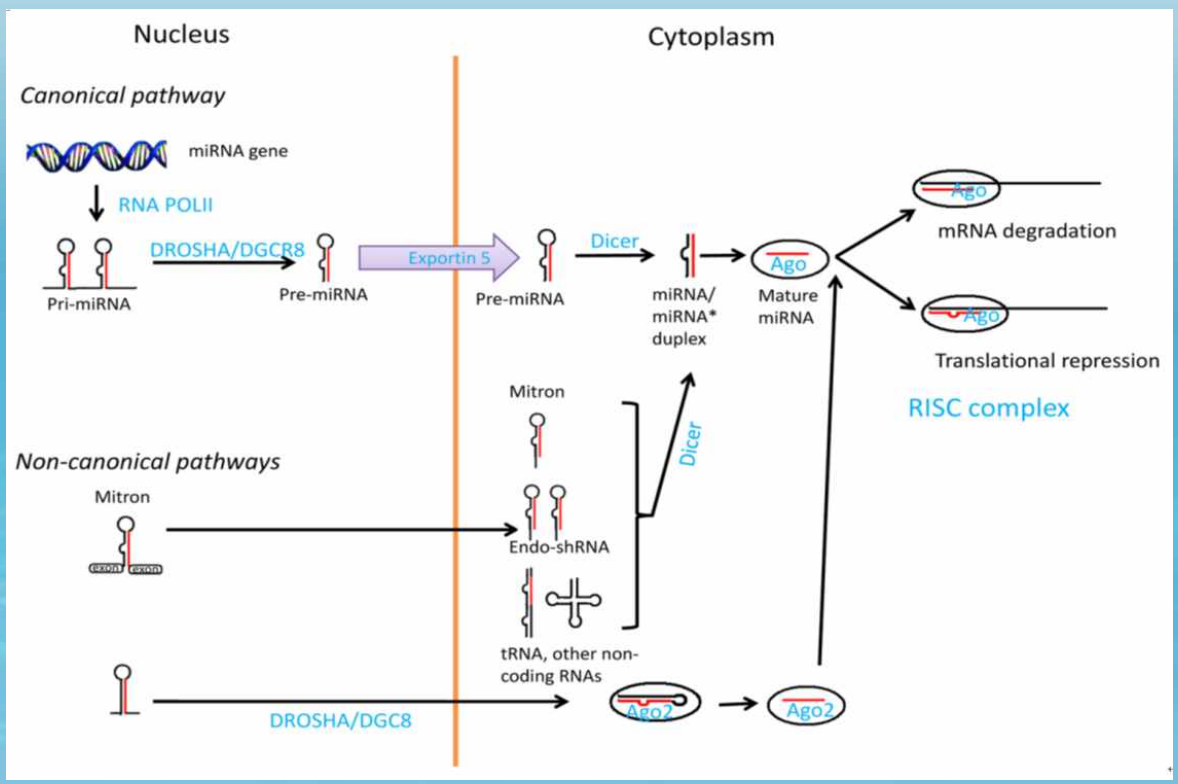

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Table of Contents

Volume 6 Number 1 January 2016

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Extracellular Micro-RNAs in Health and Disease: Basic Science, Biogenesis and Release

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Abstract

Small non-protein coding micro-RNAs are regularly exported out of cells, both in health and disease. More than ninety percent of extracellular miRNAs are associated with lower-molecular-mass complexes bound to Argonaute 2 (Ago2), nucleophosmin-1 (NPM1) and high density lipoproteins (HDL), whereas the rest (~10%) are membrane-vesicle-encapsulated within exosomes, shedding microvesicles and apoptotic bodies. Regardless of the debate of the nature of circulating miRNA as byproducts of routine cell activities or mediators of cell-cell communication, proper understanding of the molecular behaviors of miRNA in health and disease, is expected to open a new gate for the discovery of new diagnostic tools and possibly therapeutic implementation in the near future.

Keywords

Extracellular miRNAs, Extracellular Vesicles (EVs), Exosomes, Horizontal Gene Transfer (HGT), Microvesicles (MVs)

1. Introduction

Since Victor Ambros and Garry Ruvkun discovered micro-RNA (miRNAs), they revolutionized our understanding of the molecular mechanisms of cell function [1]. Functional studies indicate that miRNAs participate in the regulation of almost every cellular process, and are intrinsically associated with much of human pathology

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[2]. MiRNAs are endogenously produced, short non-coded RNAs of 21 - 25 nucleotides that are post-transcriptional component of gene regulatory network that modulate the precise amounts of proteins expressed in all cell, frequently by mRNA translation repression or less often by mRNA cleavage or degradation [2] [3].

Recently, it was also shown that mRNA may up-regulate the expression of their target genes as well [4] [5]. It is well documented that a single miRNA can influence hundreds of mRNA gene transcripts and thereby, it has been implicated as a key player in virtually all cell processes [5]-[8]. While the majority of miRNAs are found intra-cellular, a virtual number have been detected outside cells, including various body fluids (*i.e.* saliva, urine, breast milk, seminal plasma, tears, amniotic fluid, colostrum, bronchial lavage, cerebrospinal fluid, peritoneal fluid, and pleural fluid) from normal individuals [9].

Cells have protective enzymatic and non-enzymatic mechanisms against oxidative stress [10] targeting reconstitution of normal cell function. However, oxidative stress occurs when the reactive oxygen species (ROS) level overwhelms defensive mechanisms [11] [12]. The produced miRNAs under these different conditions varies accordingly. Furthermore, alterations in the level and composition of these extracellular miRNAs, as part of extracellular RNAs (exRNAs), have been well correlated with disease or injurious conditions [13]-[16], suggesting that these extracellular miRNAs can be served as diagnostic and prognostic biomarkers. The source of exRNAs can be endogenous or exogenous, including microbes and foods [17]. Recent findings show that exRNA can act as a signaling molecule, communicating with other cells and carrying genetic information from cell to cell throughout the body [1]. Circulating miRNAs are remarkably stable despite high extracellular RNase activity [9]. In addition to packing within extracellular vesicles (EVs) which are impermeable to RNases, extracellular miRNAs are also packaged in some manner to protect them against RNase digestion, through the formation of protein-miRNA complexes [18].

It is of note here to mention that horizontal gene transfer (HGT) is occurring without genomic integration. Although the study of miRNA is still in the very early stages, current research is exploring the association between various diseases and changes in the type and amount of miRNAs. These diseases include several types of cancer, neurological disorders, heart disease, kidney disease, and more [19]-[22]. This review provides an overview of the properties of extracellular miRNAs, summarizing the current theories regarding extracellular miRNA origin and function, arguing for more compelling translation of circulating miRNAs into clinical practice.

2. Biogenesis of miRNAs (Figure 1)

Non-protein coding RNAs (ncRNAs) have linked this class of nucleic acids with a large panel of biological processes, such as homeostasis, development and carcinogenesis [23]-[25]. Different classes of small RNAs continue to be discovered. There are three major classes of animal ncRNAs; microRNAs (miRNAs), short interfering RNAs (siRNAs), and Piwi-interacting RNAs (piRNAs). MiRNAs are small, non-coding RNAs commonly found intra-cellular in all tissues and function as post-transcriptional component of gene regulators. MiRNAs can be generated in a cell by either canonical pathway or non-canonical pathways [26]-[28]. The canonical pathway is Drosha (an RNase III-like protein)/DGCR8 (DiGeorge syndrome critical region gene 8) and Dicer dependent, while the non-canonical pathway may be independent of Drosha/DGCR8 or Dicer. In both pathways, miRNAs are produced via processing and editing, resulting in the formation of mature, functional miRNAs of 21 - 25 nucleotides. Canonical pathway of miRNA biogenesis is responsible for the production of the majority of the known miRNAs. In the canonical pathway, genes are usually transcribed by RNA polymerase II (Pol II) [29] [30]. Arising from intergenic or intragenic (both exonic and intronic) genomic regions. Of note, while endogenous siRNAs originate from double-stranded (ds) RNA precursors, miRNAs and piRNAs are transcribed as long single-stranded (ss) RNAs. Primary transcripts (pri-miR), fold back to form double stranded hairpin structures which are then subjected to sequential processing; first the precursor molecules (pre-miR), 80 - 120 nucleotides long, are produced in the nucleus by type III endonuclease microprocessor DROSHA and its cofactor DGCRA8, followed by their export to the cytoplasm mediated by EXPORTIN 5.

In the cytoplasm, where they are processed by another type III endonuclease, DICER, into the short “active” molecules (guide strand), while the opposite (passenger) strand is preferentially destroyed [31] [32]. MiRNAs are finally loaded onto a multi-protein complex, called RNA-induced silencing complex (RISC), which includes argonaute (Ago) proteins (in humans four Ago proteins are recognized, Ago 1 - 4), have overlapping roles in RISC formation that mediates mRNA degradation or translational repression.

In recent years, several alternative pathways of miRNA biogenesis are identified [31] [32]. Pre-miRNA mimics, endogenous short hairpin RNA (shRNA), other hairpinstructure or other non-coding RNA, can enter the

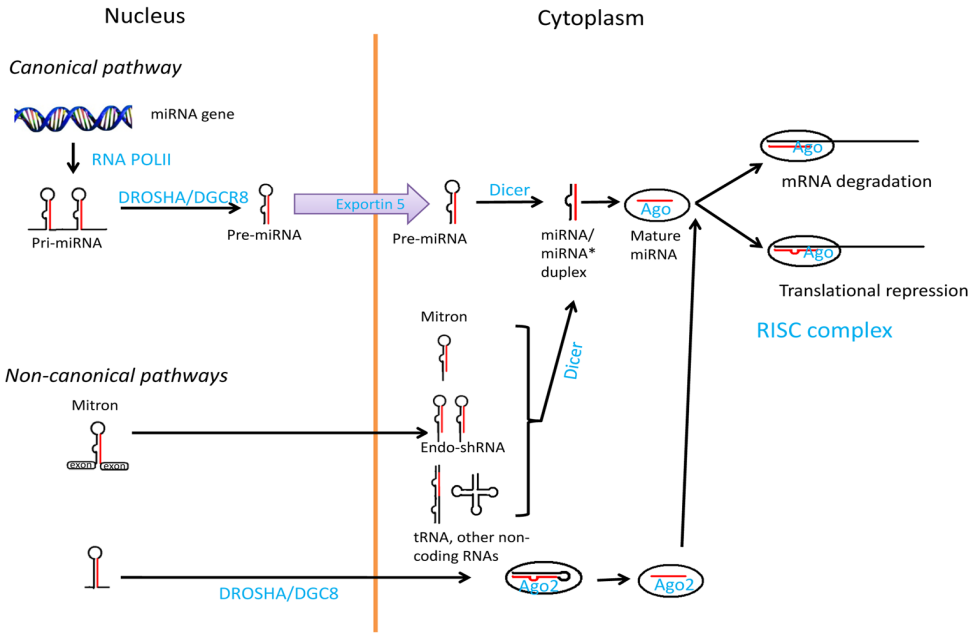

Figure 1. Biogenesis and function of microRNA (miRNA). The majority of miRNA are synthesized through the canonical pathway, in which miRNA genes are transcribed via RNA polymerase II to produce pri-miRNA (long transcripts with multiple hairpin loop structures). Pri-miRNAs are processed by the Microprocessor protein complex containing Drosha/DGCR8, resulting in generation of smaller precursor molecules called pre-miRNAs. After exported from the nucleus via Exportin-5, the pre-miRNAs are further processed via Dicer in the cytoplasm to generate short, double-stranded miRNAs. The mature miRNA direct RISC (a complex containing ago) to target gene mRNA, resulting in mRNA degradation (miRNA/mRNA with perfect complimentary sequences match in RNA). In the non-canonical pathways, miRNAs can be synthesized in either Drosha/DGCR8 or Dicer independent pathways. The short pre-miRNA like precursor can come from mitron, endo-shRNA or other noncoding RNA. In some special conditions, the precursor can be processed by Ago2 instead of Dicer to be the mature miRNA (Adopted from Qingquing Wei *et al*., 2013 [67]).

miRNA biogenesis pathway after direct processing with or without Dicer (non-canonical pathway). The mechanism(s) of piRNA biogenesis remain elusive, and are only found in animals, and specifically in the germline [31] [32].

Argonaute protein uptake of mature miRNA is thought to stabilize the guide strand, while the passenger strand is preferentially destroyed. Argonaute may preferentially retain miRNAs with many targets over miRNAs with few or no targets, leading to degradation of the non-targeting molecules. This turnover of mature miRNA is needed for rapid changes in miRNA expression profiles [33]. MiRNA biogenesis is critically regulated at the levels of epigenetic modulation, gene transcription and miRNA processing.

Many of the miRNA genes are flanked by promoter regions which are governed by specific transcription factors like Myc, p53, and hypoxia-inducible factor (HIF-1) [26]. These observations suggest that pathological challenges are pivotal in regulation of gene expression of miRNAs. In addition, global miRNA expression can also be modulated by the change of proteins or enzymes involved in miRNA processing, e.g. Drosha, exportin-5, Dicer and Ago [34].

3. Circulating microRNAs

Circulating extracellular miRNAs are remarkably stable despite high extracellular RNase activity [35]. The membranes of EVs are impermeable to RNases, therefore, attributable to the remarkable stability of vesicle encapsulated miRNA. In addition to packing within EVs, extracellular miRNAs are also packaged in some manner to protect them against RNase digestion, through the formation of protein-miRNA complexes [20] [22] [24] [25] [36] [41].

Both forms, vesicles encapsulated or protein associated, consistently revealed the presence of miRNA in almost all body fluids. Three different possibilities have been suggested to explain the different mechanisms of

miRNA secretion; i) Passive leakage from injured cells or degraded cells of short half-lives, such as platelets, ii) active secretion via Extracellular vesicles (EVs) and iii) associated with lower-molecular-mass complexes [37]-[42]. Secreted miRNAs are also shown to be remarkably stable despite the austere conditions they are subjected to in both the blood stream (RNase digestion) and during handling (e.g. extreme temperatures and pH values) [43]-[48]. It has been demonstrated that EVs-associated miRNAs represent the minority, whereas about 90% of plasma circulating miRNAs are travelling in an AGO-protein-bound form [19] [45]. Similarly, about 99% of miRNAs exported by cells in culture under normal conditions were membrane-vesicle-free and associated with AGO proteins [45]. It is feasible that the differences in isolation protocols for RNAs could account for the discrepancy in the reported distribution of extracellular miRNA between EVs and protein-bound fraction of serum.

3.1. Extracellular Vesicle-Encapsulated miRNA

A major breakthrough was the demonstration that the cargo of EVs included both mRNA and miRNA could be translated into proteins by target cells [47] [48]. Recently, analysis of RNA from EVs demonstrated that EVs contain a large variety of other small noncoding RNA species, including RNA transcripts overlapping with protein-coding regions, repeat sequences, structural RNAs, tRNA fragments, piwi RNA (piRNAs) and small interfering RNAs (siRNAs) [49] [50]. Exosomes are secretory products of endosomal origin, while, microvesicles directly bud from the plasma cell membrane [51]. The mechanism of formation of exosomes is the process of the endosomal pathway, including endocytic vesicles, early endosomes, late endosomes; also known as *multivesicular bodies* (MVBs) and finally lysosomes [52]. The pathway of multivesicular endosomes (MVEs) that are prone to fuse with lysosomes and predestined for lysosomal degradation, differ from the pathway of secretory MVEs predestined to become secreted as exosomes; *i.e.* ESCRT-dependent and ESCRT-non-dependent pathways. Endosomal sorting complex responsible for transport (ESCRT) is a four multi-protein complexes assembled within the MVEs: (ESCRT)-0, -I, -II, and -III, with associate accessory proteins (e.g., Alix and VPS4). The ESCRT-0, -I, and -II complexes recognize and sequester ubiquitinated membrane proteins at the endosomal membrane, while the ESCRT-III complex is responsible for membrane budding and actual scission of intraluminal vesicles) ILVs and exosomal release [53]-[55]. An alternative pathway, independent of the ESCRT machinery has also been described, and it includes the ceramide and sphingolipid pathway, in which the enzyme natural sphingomyelinase-2 (nSMase2) is involved inmediation of exosomal release [56] [57]. Exosomes are released by a wide spectrum of cell types and their release appears to be modulated by micro-environmental milieu and influenced by growth factors, heat shock and stress conditions, pH variations and therapy [58]-[60]. The mechanisms by which EVs affect the target cells include transfer/activation of signaling protein receptors or intercellular exchange of proteins and RNAs, which are both recruited to induce phenotypic modulation in the target cells [61].

3.2. Protein-Associated miRNA

Recently, accumulated data suggest that the majority of circulating miRNAs may not be only confined within extracellular vesicles, but a substantial fraction is also bound to protein complexes, such as high density lipoproteins (HDL), Argonaute-2 (Ago2) and nucleophosmin-1 (NPM-1). HDL bounded miRNA has been reported to be present in the plasma of healthy subjects. The uptake of HDL bounded miRNA seems to be dependent on the interplay between cholesteryl ester of HDL and the cell surface HDL receptor, named scavenger receptor class B, type I (SR-BI) [44].

Turchinovich and colleagues [43], showed that the majority of miRNAs found in human plasma, are associated with Ago2 and that the high proportion of Ago-miRNA may represent by-products of dead cells, since Ago-miRNA complexes, which are known to be present within Exosomes, are known to be extremely stable within cells [59], and it may be derived from vesicles potentially damaged during purification [74].

These findings raise indeed intriguing questions about whether these miRNAs have a different biological role and source; researchers have proposed that miRNA-protein complexes may be released into circulation as a consequence of cell lysis or necrosis, meaning that if participated in cell-cell communication this might incidentally occur under certain circumstances including cell stress and intra-cytoplasmic accumulation of unfolded or misfolded proteins. Nonetheless, it is believed that the role of these complexes has not been discovered yet.

4. MiRNA Release: Active Sorting or Byproduct of Cell Activity!

Two hypothesizes have been postulated to explain the presence of circulating extracellular miRNA, both, vesicles encapsulated or protein associated. MiRNA release either by the selective sorting system or mere byproduct of cell activity and waste disposal is yet debatable. Accumulating evidence, however, suggests that both theories can be true [43]. The hypothesis of cell activity byproduct has been claimed by the some observations; miRNA remains stable for weeks in cell lysate without enzymatic inhibition (e.g. RNases), which support the notion of simple passive release of miRNA in response to over production and cytoplasmic crowd [42]. Moreover, miRNA produced by several cell lines after three days culture under normal conditions was significantly lower than their parental cells of origin which supports this notion. Besides, the extracellular miRNA production by some cell lines is mainly (>95%) in a protein-associated state [42]. Moreover, apart from crowd and overflow release, yet, no clear indication of either active release of vesicle-free AGO2-miRNA complexes from cells or their uptake by recipient cells in mammals [43]. In contrast, miRNAs entrapped within microvesicles can horizontally transfer gene information to recipient cells, modulate gene expression and trigger functional effects [62] [63]. The exact mechanisms by which cells scission/release EVs containing miRNAs remains incompletely understood; however, the release is multifactorial and modulated particularly by extracellular signals [64]. Microvesicles are released by direct budding from the plasma cell membrane, whereas exosomes release process might be ceramide enzyme nSMase2-dependent or nondependent machinery [65]. However, exosome release is blocked by inhibiting nSMase2 (neutral sphingomyelinase 2) and interestingly, inhibition of nSMase2 actually increases the export of miRNAs by HDL [65], suggesting two different pathways to exert the same action, meaning, two distinct mechanisms and/or competition in the export pathways. Moreover, the ESCRT-III complex is responsible for membrane budding and actual scission of intraluminal vesicles (ILVs) and exosomal release [53]-[55]. The activation of nSMase2 enzyme and ESCRT-III complex and their involvement in the release of vesicles might be a response to the crowd and overflow proposition. This means that, in case of crowd and overflow, the cell endosomal pathway is unable to cope with the abrupt increase in the need for degradation through the endocytic pathway, hence activation of an alternative pathway to eliminate and secrete these contents outside the cell. Other observations have been supporting the hypothesis of active sorting; high EVs miRNA level than that contained in the parental cells which may be explained by the observation that certain miRNAs can be lost during extraction from samples [66]. The differences in the blood collection protocols willimpact the comparison between extracellular versus intracellular miRNA profiles expressed in different cell types [67]-[71].

5. Function of miRNA

Each class of small RNAs binds to a member of the Argonaute (Ago) family of proteins. The Argonaute protein family includes eight members (*Ago* 1 - 4, present in all mammalian cells and *Piwi* 1 - 4, found in the germ line and hematopoietic stem cells) in the human genome [68]. The siRNAs and miRNAs associate the Ago clade, whereas piRNAs associate the Piwi clade [69]. The Ago protein bound to the small RNA comprises the RNA-induced silencing complex (RISC). The RISCs containing miRNAs are primarily target messenger RNAs (mRNAs) by either complete complementarity causing degradation of the mRNAs or incomplete complementarity, causing translation repression (slowing or preventing mRNA translation) [70], a combination of the two is hotly debated. However, the balance between repressed and degraded mRNA might play an integral role as post-transcriptional component of gene regulatory network that modulate the precise amounts and type of proteins expressed in cells. MiRNA is a crucial player in gene regulation through different mechanisms of actions mostly controlling gene transcription and translation processes [69]. Recently, miRNA has been also reported to induce histone modifications methylation of the DNA promotor sites and thus, ameliorating the target gene expression [70].

6. Clinical Applications of Extracellular miRNA

The search for novel, early disease predictors represent a current and a permanent challenge in disease recognition. MiRNAs offer a new class of biologically active molecules that contribute many disease processes [71]. Accumulating evidences showed that circulating extracellular miRNA, both, vesicles encapsulated or protein-associated can serve as disease biomarkers [72]. Some points need to be cleared to maximize the benefit

and minimize the pitfalls. Cell contribution in the disease state within the same organ varies among different cells of the same organ. This variability may be attributed to the stress phase, which differs among cells even within the same organ. RNA microarray profiling of intracellular miRNA may only reflect the stress phase of a particular cell. On the other hand, extracellular circulating miRNA represents the final net result of miRNAs that produced by most of involved cells. Besides, vesicles are subjected to shearing force associated with centrifugation and filtration that might alter its miRNA contents [72]. But the possibility of other distant organ miRNA release that might alter the interpretation of the results is still there. Confounding biological and technical factors, such as age or gender of patients as well as storage conditions and processing protocols, are essential in order to judge the value of miRNAs as biomarkers [72]. Besides, identification of a set of extracellular miRNAs rather than single up or down regulated miRNA against intracellular protein microarray would be more reliable biomarker for a specific disease. Recently, not only miRNA are recognized as disease biomarkers, but also miRNAs signatures are increasingly recognized as valuable biomarkers for a differential diagnosis in clinically overlapping diseases such as non-ischemic systolic heart failure versus acute myocardial infarction [73].

Nishimura N. and his collaborators addressed the combination of miRNA and siRNA in nanoliposomal particles to target oncogenic pathways altered in ovarian cancer. Combined targeting of the Eph pathway using *EphA*2 -targeting siRNA and the tumor suppressor miR-520d-3p exhibits remarkable therapeutic synergy and enhanced tumor suppression *in vitro* and *in vivo* compared with either monotherapy alone [74].

Recently, miR-423-5p, miR-18b-3p, miR-129-5p, miR-1254, miR-675, and miR-622) were reported to be elevated in patients with heart failure, with miR-423-5p positively correlated with N-terminal pro-brain natriuretic peptide (NT-proBNP) levels and most strongly related to the clinical diagnosis of heart failure [75]. Besides, plasma levels of miR-208b and miR-499 both have been highly associated with AMI. Also, it was demonstrated that measuring miR-1 in plasma is a good approach for blood-based detection of human AMI [76]. Circulating miR-1 is significantly increased in the blood of AMI patients compared to non-AMI subjects and were positively correlated with serum CK-MB (Creatine kinase-MB) [77].

Kato and collaborators recently show that miR-192 levels increase significantly in glomeruli isolated from streptozotocin-injected diabetic mice as well as diabetic db/db mice, in parallel with increased TGF-β1 and collagen 1a2 (Col1a2) levels [78]. Upregulation of renal miR-192 during diabetic kidney diseases is also found in db/db mice, type 2 diabetes rat, and whole blood samples of type 2 diabetes patients [79].

7. Conclusions

The fact that diverse diseases in different organ systems (liver, kidney, pancreas, heart, lung and skin) may end with fibrotic changes suggests common pathogenic pathways. These pathways are orchestrated by complex activities within different cells in which specific molecular pathways and gene expression have emerged. These specific molecules are cell specific (relates to the stress phase of cells involved), rather than organ specific. Current researches are exploring the association between various diseases and changes in miRNAs as part of molecular pathways activation. The activation of molecular pathways of cell stress in different diseases seems to be more or less similar regardless the organ involved in a specific disease; resulting in cellular dysfunction, parenchymal scarring and ultimately organ failure. The clinical presentation of any disease can be explained as "*functional expression of cell disruption*" depending on the cell mass involved in this response.

As all living organisms should have a facility to get rid of their metabolic wastes. Exosomes, microvesicles, apoptotic bodies and protein associated nucleoproteins might be some forms of cell excreta. Two hypothesizes have been postulated to explain the presence of circulating extracellular RNAs, both, vesicles encapsulated or protein associated. RNAs release either by the selective sorting system or mere byproduct of cell activity and waste disposal is yet debatable. Accumulating evidence, however, suggests that both theories can be true. Extracellular nucleoproteins consist of a variety of factors among which miRNA is seemingly of particular importance in response to molecular pathways activation. Yet, we treat most of diseases after the onset of the functional disturbances, while thorough understanding of the molecular cell biology will enable us to diagnose and hence treat cells far before the onset of symptoms and signs by targeting molecular cell disruption in which miRNA is a pivotal player.

The response of cells to stress, which can be physical, chemical or biological stressor(s), is a series of biological events that facilitate and promote counteraction and adaptation to qualify these cells to survive. Cell adaptation can be categorized into five major types including atrophy, hypertrophy, hyperplasia, dysplasia and metap-

lasia. The adaptation may be physiologic or pathologic. In case of failure of adaptation, cells will proceed to a state of cell death, through undergoing autophagy, apoptosis and necrosis. From cell adaptation to cell death, the process of fibro-genesis is activated to modulate the final anatomical and physiological outcome of the tissue in reaction to stress. Duration, severity and frequency of exposure to stressors are detrimental for the nature of cell response to stress. Correlation between exRNA profiles in various adaptation mechanisms has not yet been thoroughly investigated. This profile will expedite the discovery of complex panel of biomarkers to monitor specific disease states along the complete pathogenic pathway for prediction, initiation, development, diagnosis, progression, regression, and treatment efficacy of the disease.

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Conflict of Interest

We, the authors of this review, declare that there is no conflict of interest that could be perceived as prejudicing impartiality of the research. We fully declare that no financial or other potential conflict of interest.

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Genetic Diversity of Maize Accessions (*Zea mays* L.) Cultivated from Benin Using Microsatellites Markers

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Abstract

Maize (*Zea mays* L.) is the major cereal cultivated in Benin and it is important to know its genetic diversity to improve the yield. The genetic markers of important traits are evaluated in order to improve the maize inbred lines. The aim of this study was to evaluate the genetic diversity of Benin's maize accessions by SSR marker. Thus, one hundred eighty seven maize accessions from three areas (South, Center and North) were analyzed using three SSR markers. A total of 227 polymorphic bands were produced and showed high genetic diversity (Shannon index = 0.51). The polymorphic information content (PIC) values for the SSR loci ranged from 0.58 to 0.81, with an average of 0.71. Genetic distance-based UPGMA dendrogram showed a genetic differentiation between accessions and they were grouped into four clusters in each area. This work provides necessary information that can be used not only to improve the maize production and conservation but also to better manage genetic species resources in Benin.

Keywords

SSR, PCR, Molecular Characterization, *Zea mays* L., Benin

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1. Introduction

Maize is the major cereal growing in the humid tropics and sub-Saharans Africa climate [1]. It is a changeable cereal classified third in world cereal production after wheat and rice [2]. Maize represents an actual source of consumption and income for millions of people in several countries [3]. This cereal belongs to the Andropogoneae tribe, Panicoideae subfamily and Poaceae family [4]. Five species are included in the genus Zea and largely has 2n = 20 chromosomes (except Zea perennis, 2n = 40) [5].

In Benin, maize occupies about 82% of total cereals cultivated area and represents about 84% of national cereal production. Thus, this cereal appears as essential product in Benin [6] and is characterized by large range of varieties (improved and local) managed by producers themselves [7]. Despite its enormous potential, Benin's agriculture is struggling to ensure sustainable food security due to constraints such as low yield [7]. So, researches that contribute to improve of the maize production yields are necessary to lift this constraint. One of the main contributions will be the development of improved varieties, among local maize resources, that meet the expectations of producers. Indeed, the local resources have a very significant phenotypic variability and genetic diversity and constitute an essential component of food security, as they provide the raw material used by breeders to improve the quality and productivity of maize. It is therefore necessary to know the genetic characteristics maize usually grown in Benin. For such characterization, the use of molecular markers provided an opportunity to analyze large-scale of maize populations [8] like previously used to study the structure of plants genetic variation [9] [10].

Various molecular genetic markers such as Restriction Fragment Length Polymorphism (RFLP) [11], Amplified Fragment Length Polymorphism (AFLP) [12], Inter Simple Sequence Repeats (ISSR) [13] and Simple sequence repeats (SSR) [14] were reported to be used in the molecular characterization of various plant genetic resources. Meanwhile, the SSR loci are reported to be highly polymorphic for the basic number of repeat units between species and especially among individuals within species and populations [15]. Widely used in the construction of the genetic map of the human genome, the SSR markers were used in the mapping of the plant genome [16]. Thus, in many plants, microsatellites are known to be more effective in genetic characterization and better indicated for structuring of genetic diversity studies [17] [18]. So, considering their interest in genetics, they are known to be neutral markers, co-dominant, extremely polymorphic and distributed throughout the genome [19]. Then, the aim of this study was to analyze with SSRs markers, the genetic polymorphism that may exist between different corn accessions collected in Benin.

2. Material and Methods

2.1. Plant Materials

Two hundred thirty three accessions of maize from seven agroecological zone in Benin were used in this study (**Figure 1** and **Table 1**). This collection includes the improved cultivars and local cultivars acquired from National Agriculture Research Institute of Benin (INRAB) [20]. In this study, among the two hundred thirty three accessions collected, one hundred eighty seven accessions have germinated.

2.2. DNA Extraction

Maize accessions were grown in the greenhouse. The single plant (3 weeks old) was taken from each accession and stored at −80˚C. Single-plant samples were ground to powder in liquid nitrogen using a mortar and pestle. A total genomic DNA was extracted as described previously [21].

2.3. SSR Analysis

Three SSR primers of maize (**Table 2**) were selected from previous studies [22]. The total volume of PCR mixture was 20 µl containing 10 µl of master mix [AccuStart II PCR ToughMix (2×)], 2.5 µl template DNA, 1 µl of each primer (Forward and reverse) and 5.5 µl water.

The PCR reaction was performed in a thermal cycler (BIO-RAD; T100TM) using an initial 94˚C denaturing step for 3 min followed by 34 cycles of [denaturation at 94˚C for 30 s, annealing for 30 s at the primer's annealing temperature, extension at 72˚C for 1 min 20 s] and a final extension at 72˚C for 5 min.

2.4. Data Analysis

The presence (1) or absence (0) of a PCR amplified SSR markers band were coded. The data base was then regis-

Table 1. Identification numbers, Site of collection, agroecological zone of maize accessions collected in Benin.

No.	ID	Locality	Agroecological zone	No.	ID	Locality	Agroecological zone	No.	ID	Locality	Agroecological zone
1	Zm1	vidjinan	Zone VIII	**36**	Zm36	Houèglè	Zone VII	**71**	Zm71	Hèkpè	Zone VI
2	Zm2	vidjinan	Zone VIII	**37**	Zm37	Ayahonou	Zone VII	**72**	Zm72	Sènouhoué	Zone VI
3	Zm3	vidjinan	Zone VIII	**38**	Zm38	Niaouli	Zone VI	**73**	Zm73	Sènouhoué	Zone VI
4	Zm4	Ayihounzo	Zone VIII	**39**	Zm39	Covè	Zone VI	**74**	Zm74	Sènouhoué	Zone VI
5	Zm5	Sèmé	Zone VIII	**40**	Zm40	Covè	Zone VI	**75**	Zm75	Sènouhoué	Zone VI
6	Zm6	Dossivi	Zone VIII	**41**	Zm41	Avlimè	Zone VI	**76**	Zm76	Agohoué-balimey	Zone VI
7	Zm7	Kodé	Zone VIII	**42**	Zm42	Avlimè	Zone VI	**77**	Zm77	Agohoué	Zone VI
8	Zm8	Kodé	Zone VIII	**43**	Zm43	Avlimè	Zone VI	**78**	Zm78	Ahogbéya	Zone VI
9	Zm9	Kodé	Zone VIII	**44**	Zm44	Domado	Zone VI	**79**	Zm79	Ahogbéya	Zone VI
10	Zm10	Kodé	Zone VIII	**45**	Zm45	Gbédji	Zone VI	**80**	Zm80	Ahogbéya	Zone VI
11	Zm11	Kodé	Zone VIII	**46**	Zm46	Gbédji	Zone VI	**81**	Zm81	Ahogbéya	Zone VI
12	Zm12	Kakanitchoé	Zone VIII	**47**	Zm47	Gbédji	Zone VI	**82**	Zm82	Ahogbéya	Zone VI
13	Zm13	Kakanitchoé	Zone VIII	**48**	Zm48	Gbédji	Zone VI	**83**	Zm83	Ahogbéya	Zone VI
14	Zm14	Atanka	Zone V	**49**	Zm49	Lohounvodo	Zone VIII	**84**	Zm84	Ahogbéya	Zone VI
15	Zm15	Atanka	Zone V	**50**	Zm50	Lohounvodo	Zone VIII	**85**	Zm85	Sèglahoué	Zone VI
16	Zm16	Atanka	Zone V	**51**	Zm51	Atikpéta	Zone VIII	**86**	Zm86	Sèglahoué	Zone VI
17	Zm17	Kpankou	Zone V	**52**	Zm52	Djéhadji	Zone VIII	**87**	Zm87	Sèglahoué	Zone VI
18	Zm18	Kpankou	Zone V	**53**	Zm53	Adjaïgbonou	Zone VII	**88**	Zm88	Sèglahoué	Zone VI
19	Zm19	Kpankou	Zone V	**54**	Zm54	Adjaïgbonou	Zone VII	**89**	Zm89	Gbénounkochihoué	Zone VI
20	Zm20	Vloko	Zone V	**55**	Zm55	Adjaïgbonou	Zone VII	**90**	Zm90	Gbénounkochihoué	Zone VI
21	Zm21	Issaba	Zone VII	**56**	Zm56	Adjaïgbonou	Zone VII	**91**	Zm91	Gbénounkochihoué	Zone VI
22	Zm22	Issaba	Zone VII	**57**	Zm57	Adjaïgbonou	Zone VII	**92**	Zm92	Massi	Zone VII
23	Zm23	Ayogo	Zone VI	**58**	Zm58	Adjaïgbonou	Zone VII	**93**	Zm93	Massi	Zone VII
24	Zm24	Sédjè	Zone VI	**59**	Zm59	Adjaïgbonou	Zone VII	**94**	Zm94	Atoungon	Zone VII
25	Zm25	Houezeto	Zone VI	**60**	Zm60	Gnamamé	Zone VII	**95**	Zm95	Atoungon	Zone VII
26	Zm26	Sédjè	Zone VI	**61**	Zm61	Gnamamé	Zone VII	**96**	Zm96	Hlanhonou	Zone VII
27	Zm27	Glégbodji I	Zone VI	**62**	Zm62	Gnamamé	Zone VII	**97**	Zm97	Hlanhonou	Zone VII
28	Zm28	Glégbodji I	Zone VI	**63**	Zm63	Gnamamé	Zone VII	**98**	Zm98	Hlanhonou	Zone VII
29	Zm29	Glégbodji I	Zone VI	**64**	Zm64	Banigbé	Zone VII	**99**	Zm99	Kotokpa	Zone VII
30	Zm30	Dohinhonko	Zone VI	**65**	Zm65	Banigbé	Zone VII	**100**	Zm100	Koussoukpa	Zone VII
31	Zm31	Dohinhonko	Zone VI	**66**	Zm66	Banigbé	Zone VII	**101**	Zm101	Koussoupka	Zone VII
32	Zm32	Dohinhonko	Zone VI	**67**	Zm67	Atchouhoué	Zone VI	**102**	Zm102	Agoïta	Zone VII
33	Zm33	Agonmey	Zone VII	**68**	Zm68	Atchouhoué	Zone VI	**103**	Zm103	Dohouimey	Zone V
34	Zm34	Agonmey	Zone VII	**69**	Zm69	Atchouhoué	Zone VI	**104**	Zm104	Honhoun	Zone V
35	Zm35	Agonmey	Zone VII	**70**	Zm70	Atchouhoué	Zone VI	**105**	Zm105	Honhoun	Zone V
106	Zm106	Lantêdié	Zone V	**143**	Zm143	Kpari	Zone V	**180**	Zm180	Biro	Zone III
107	Zm107	Lantêdié	Zone V	**144**	Zm144	Boue	Zone V	**181**	Zm181	Yambérou	Zone II
108	Zm108	Lantêdié	Zone V	**145**	Zm145	Boue	Zone V	**182**	Zm182	Yambérou	Zone II

Continued

109	Zm109	Lantêdié	Zone V	**146**	Zm146	Boue	Zone V	**183**	Zm183	Yambérou	Zone II
110	Zm110	Fonkpodji	Zone V	**147**	Zm147	Boue	Zone V	**184**	Zm184	Kpako soro kpika	Zone II
111	Zm111	Fonkpodji	Zone V	**148**	Zm148	Gounin	Zone III	**185**	Zm185	Kpako soro kpika	Zone II
112	Zm112	Agoua	Zone V	**149**	Zm149	Gounin	Zone III	**186**	Zm186	Kpako soro kpika	Zone II
113	Zm113	Boobè	Zone V	**150**	Zm150	Gounin	Zone III	**187**	Zm187	Yaroubosso	Zone II
114	Zm114	Boobè	Zone V	**151**	Zm151	Gounin	Zone III	**188**	Zm188	Yaroubosso	Zone II
115	Zm115	Agoua	Zone V	**152**	Zm152	Gounin	Zone III	**189**	Zm189	Yaroubosso	Zone II
116	Zm116	Pira	Zone V	**153**	Zm153	Gounin	Zone III	**190**	Zm190	Ounet	Zone II
117	Zm117	Aguélé	Zone V	**154**	Zm154	Bounyérou	Zone III	**191**	Zm191	Ounet	Zone II
118	Zm118	Aguélé	Zone V	**155**	Zm155	Bounyérou	Zone III	**192**	Zm192	Ounet	Zone II
119	Zm119	Azongnihogon	Zone V	**156**	Zm156	Bounyérou	Zone III	**193**	Zm193	Ounet	Zone II
120	Zm120	Azongnihogon	Zone V	**157**	Zm157	Bounyérou	Zone III	**194**	Zm194	Gomparou	Zone II
121	Zm121	Azongnihogon	Zone V	**158**	Zm158	Bounyérou	Zone III	**195**	Zm195	Gomparou	Zone II
122	Zm122	Ayéladjou	Zone V	**159**	Zm159	Banhounkpo	Zone III	**196**	Zm196	Zougoupantrossi	Zone II
123	Zm123	Ayéladjou	Zone V	**160**	Zm160	Banhounkpo	Zone III	**197**	Zm197	Badou	Zone II
124	Zm124	Koutoukou	Zone V	**161**	Zm161	Banhounkpo	Zone III	**198**	Zm198	Badou	Zone II
125	Zm125	Pounga	Zone V	**162**	Zm162	Banhounkpo	Zone III	**199**	Zm199	Badou	Zone II
126	Zm126	Atchakpa	Zone V	**163**	Zm163	Banhounkpo	Zone III	**200**	Zm200	Badou	Zone II
127	Zm127	Atchakpa	Zone V	**164**	Zm164	Sakarou	Zone III	**201**	Zm201	Badou	Zone II
128	Zm128	Atchakpa	Zone V	**165**	Zm165	Sakarou	Zone III	**202**	Zm202	Badou	Zone II
129	Zm129	Atchakpa	Zone V	**166**	Zm166	Sakarou	Zone III	**203**	Zm203	Bagou	Zone II
130	Zm130	Atchakpa	Zone V	**167**	Zm167	Sakarou	Zone III	**204**	Zm204	Warra	Zone II
131	Zm131	Gbanlin	Zone V	**168**	Zm168	Sakarou	Zone III	**205**	Zm205	Warra	Zone II
132	Zm132	Gbanlin	Zone V	**169**	Zm169	Ponaga	Zone III	**206**	Zm206	Partago	Zone IV
133	Zm133	Yaoui	Zone V	**170**	Zm170	Ponaga	Zone III	**207**	Zm207	Partago	Zone IV
134	Zm134	Yaoui	Zone V	**171**	Zm171	Ponaga	Zone III	**208**	Zm208	Partago	Zone IV
135	Zm135	Kassehlo	Zone V	**172**	Zm172	Ponaga	Zone III	**209**	Zm209	Partago	Zone IV
136	Zm136	Kpassatona	Zone V	**173**	Zm173	Ganrou	Zone III	**210**	Zm210	Monmongou	Zone IV
137	Zm137	Kpassatona	Zone V	**174**	Zm174	Ganrou	Zone III	**211**	Zm211	Sérou	Zone IV
138	Zm138	Kpassatona	Zone V	**175**	Zm175	Ganrou	Zone III	**212**	Zm212	Angara	Zone IV
139	Zm139	Kpassatona	Zone V	**176**	Zm176	Kassakpéré	Zone III	**213**	Zm213	Angara	Zone IV
140	Zm140	Kpassatona	Zone V	**177**	Zm177	Kassakpéré	Zone III	**214**	Zm214	Firou	Zone II
141	Zm141	Kpari	Zone V	**178**	Zm178	Kassakpéré	Zone III	**215**	Zm215	Firou	Zone II
142	Zm142	Kpari	Zone V	**179**	Zm179	Biro	Zone III	**216**	Zm216	Firou	Zone II
217	Zm217	Kaobagou	Zone II	**223**	Zm223	Yédékanhoun	Zone IV	**229**	Zm229	Holli	Zone IV
218	Zm218	Kaobagou	Zone II	**224**	Zm224	Yédékanhoun	Zone IV	**230**	Zm230	Holli	Zone IV
219	Zm219	Djoléni	Zone II	**225**	Zm225	Boliféri	Zone IV	**231**	Zm231	Kotari	Zone IV
220	Zm220	Pikéré	Zone II	**226**	Zm226	Boliféri	Zone IV	**232**	Zm232	Kotari	Zone IV
221	Zm221	Komgourou	Zone II	**227**	Zm227	Boliféri	Zone IV	**233**	Zm233	Kotari	Zone IV
222	Zm222	Yédékanhoun	Zone IV	**228**	Zm228	Holli	Zone IV				

Zm: Zea mays; Zone II: Cotton zone of Northern Benin; Zone III: Food area south Borgou; Zone IV: Area west Atacora; Zone V: Cotton zone of central Benin; Zone VI: Land area bar; Zone VII: Suction zone; Zone VIII: Fishery Zone.

Table 2. Characteristics of SSR primers used in this study.

Markers Name	Bin[1]	Motif	Sequence (5'-3')
Umc1222	1.01	(AG) 20	For: CTCAGAACAGAAGCCATCAAAAGC Rev: CGTCTTCGTGAGAGACATCCTGT
Umc 1335	1.06	(AG) 24.	For: ATGGCATGCATGTGTTTGTTTTAC Rev: ACAGACGTCGCTAATTCCTGAAAG
Umc 1327	8.01	(GCC) 4	For: AGGGTTTTGCTCTTGGAATCTCTC Rev: GAGGAAGGAGGAGGTCGTATCGT

[1]: Position in the chromosome; For: Forward; Rev: Reverse.

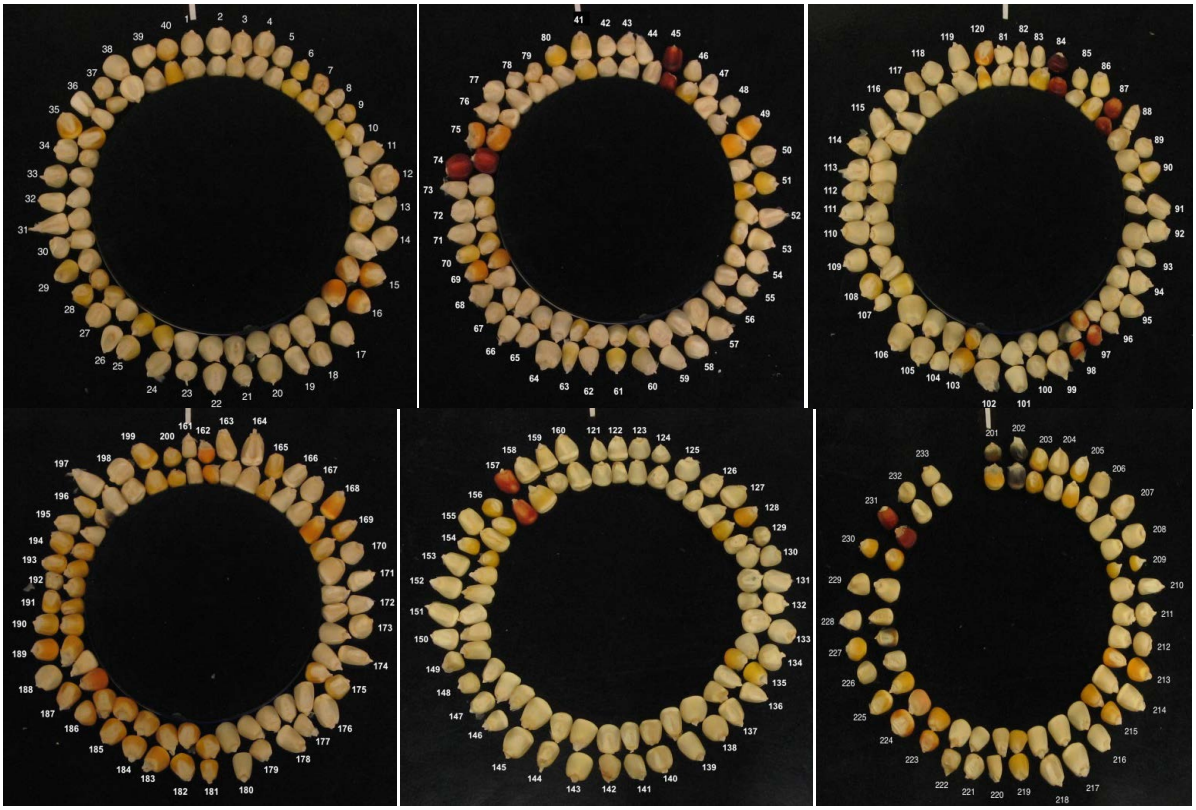

Figure 1. Picture showing the phenotypical diversity of maize accession collected in Benin.

tered in an MS Excel spreadsheet in order to generate the analysis matrix. Genetic diversity parameters such as Polymorphism Information Content (PIC) as previously describe by Anderson *et al.* [23]; polymorphism rate (P), number of alleles (Na), expected heterozygosity (He) and Shannon's phenetic index (H) were estimated according to the method used by Adoukonou-Sagbadja *et al.* [9].

Cluster analysis by Un-weighted Pair Group Method using Arithmetic Averages (UPGMA) and principal coordinate analysis (PCoA) were performed to identify genetic variation patterns among the maize genotypes using DarWin and NTSYSpc (2.2) data bases software respectively.

3. Results

3.1. Classification of the Maize Accessions According to Germination Time

Figure 2 shows the germination percentage of maize accessions according to the number of day after seeding. Analyze of this figure shows that the percentage of germination varied not only according the number of day after seeding but also according to the zone. Thus, in south (**Figure 2(a)**) the accessions can be grouped in two clusters. The accessions of cluster I (63%) have a middle germination time (three or four days after seeding).

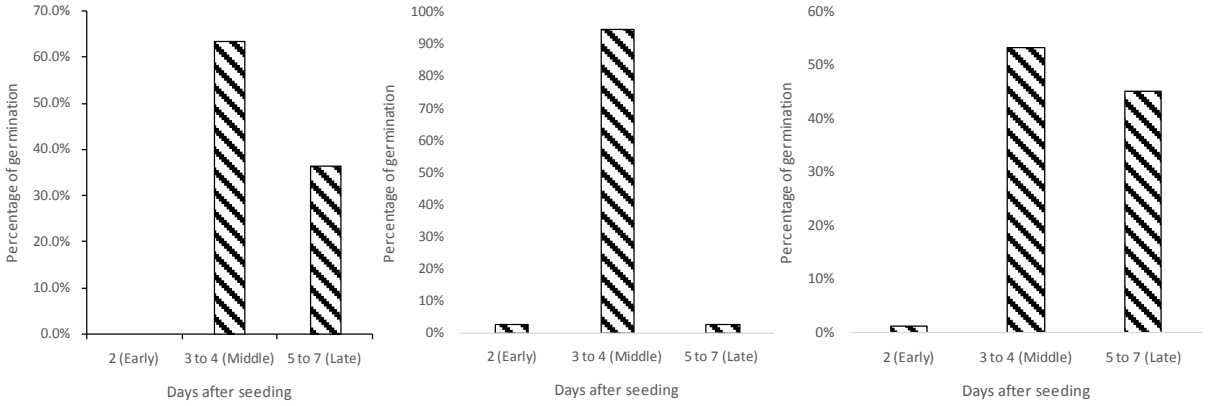

Figure 2. Percentage of germination according to the day after seeding of maize accessions. (a) Southern; (b) Center; (c) Northern.

The cluster II, regroup 37% of accessions, have a late germination time (≥ five days after seeding).

As for maize accessions of center, they can be classified in three clusters according to the germination time (**Figure 2(b)**). The cluster I contain the accessions of maize that have early germination time (two days after seeding). This cluster regroups 3% of the whole accessions. The cluster II contain 93% of accessions and was characterized by a middle germination time (three to four day after seeding). The cluster III maize accessions (4%) have a late germination time (≥ five days after seeding).

Figure 2(c) shows the classification of north maize accessions in three clusters. The first cluster contains the accessions that have early germination time (two days after seeding). The accessions of cluster II have a middle germination time (three to four day after seeding) and the cluster III was characterized by a late germination time (≥ five days after seeding).

3.2. SSR Polymorphism

The SSR markers selected to analyze the genetic diversity of the maize accessions displayed different characteristic profiles. Thus, different numbers of polymorphic bands, percentage of polymorphism, Polymorphism Information Content (PIC), and expected heterozygosis have been generated using the SSR markers (**Table 3**). All microsatellite markers used were found to be polymorphic, in other words a loci polymorphic rate of 100% was observed and the number of bands generated by each marker varied from 58 to 102 (76 a mean value). The level of polymorphism ranged from 25.33% to 44.54%. The discriminating power of each primer pair, estimated by the value of the PIC varied between 0.58 and 0.81 with an average rate of 0.71% for all SSRs analyzed.

3.3. Genetic Differentiation

Among the 227 distinct scored bands (~2 bands/accessions); 41% (n = 92) were recorded for south accessions, 23% (n = 52) for Center's accessions and 36% (n = 83) for the North accessions (**Table 4**). There were no specific bands belonging to accessions of the same production area. The South and North's accessions showed a high polymorphism and the number of accessions per zone had no effect on the percentage of polymorphism. To end, the Shannon index varied between 0.49 and 0.53 with an average of 0.52 for all accessions.

3.4. Genetic Relationship and Cluster Analyses

Genetic relationships among maize cultivars were determined by the Unweighted Pair Group Method with Arithmetic mean (UPGMA) using the Nei distances [24]. This method showed a dendrograms profiles of the maize accession respectively from Southern, Central and Northern Benin. The analysis of dendrograms showed the heterogeneity between local and improved accession in each area (**Table 5** and **Figures 3-5**).

The first dendogram shows the threshold of 14% similarity, the southern cultivars were grouped into four clusters (**Figure 3**). The clusters I and II were composed of as many individuals and contain both local and improve cultivars collected from South Benin areas. Cultivars of cluster I and II have a large height of plant and

Table 3. Number of scored polymorphic bands, percentage of polymorphism, estimated PIC, and expected heterozygosis (He) of three SSR markers.

Loci	Number of scored polymorphic	Polymorphism %	PIC	He
Zm1	69	30.13	0.75	0,46
Zm2	102	44.54	0.58	0.50
Zm3	58	25.33	0.81	0.43

Zm: *Zea mays*; He: Heterozygosis expected, PIC: Polymorphism Information Content.

Table 4. Genetic diversity of cultivars based on maize growing in Benin.

Area	Number of cultivars	Number of Loci amplified	Polymorphism (%)	Shannon index
Southern	74	92	45.53	0.53
Center	38	52	22.91	0.49
North	75	83	36.56	0.53
Total	187	227	100	

Table 5. Result showing the characteristic of the dendrogram cluster of different area of Benin.

Area of Benin	Clusters	Characteristic of cluster
South-Benin	Cluster 1	Large height plant, good husk cover, late flowering and large height cob insertion.
	Cluster 2	Large height plant, good husk cover and late flowering
	Cluster 3	Medium husk cover and average height plant.
	Cluster 4	Small height plant, bad husk cover, middle germination time and early flowering
Center-Benin	Cluster 1 (A and B)	Small height, bad husk cover, early germination time and early flowering
	Cluster 2 (C and D)	Medium height and medium husk cover.
North-Benin	Cluster 1	Large height plant, good husk cover and late flowering.
	Cluster 2	Medium height and bad husk cover.
	Cluster 3	Small height, bad husk cover, late germination time and early flowering.
	Cluster 4	Medium height, bad husk cover and late flowering.

have a good husk cover with late flowering only that the plants cluster I were larger. Cluster III consisting of fourteen accessions have a mediumhusk cover and have an average height of plant. Cluster IV composed of twenty-seven accessions were small height, have a bad husk cover, and have a middle germination time but unlike cluster I and II plants early flowering (**Table 5**).

The second dendogram shows the threshold of 20% similarity, the cultivars collected from center Benin were grouped into two clusters and each cluster into two sub-clusters (**Figure 4**). Cluster I with its two sub-clusters (A and B) is consists of eighteen accessions and different from the cluster II to the threshold of 15%. This group consists of cultivars from all villages of Central corn production area. The sub-cluster A composed of ten accessions are morphologically different from those of the sub-cluster B. The plants of this cluster were small height and have a bad husk cover, early flowering and early germination time. The cluster II as consisting of two sub-clusters (C and D) is composed of 21 accessions. The plants of this cluster are medium height and have a medium husk cover (**Table 5**).

The CAH analysis based on the Euclidean distance computed using the UPGMA method clustered the north accessions into four clusters at the similarity threshold of 0.75 (**Figure 5**). The cluster I different of other clusters to 0.60 thresholds is composed of 19 local and improved collected from North. Plants of this group were large height and have a good husk cover but late flowering. Twenty accessions composed the cluster II. This

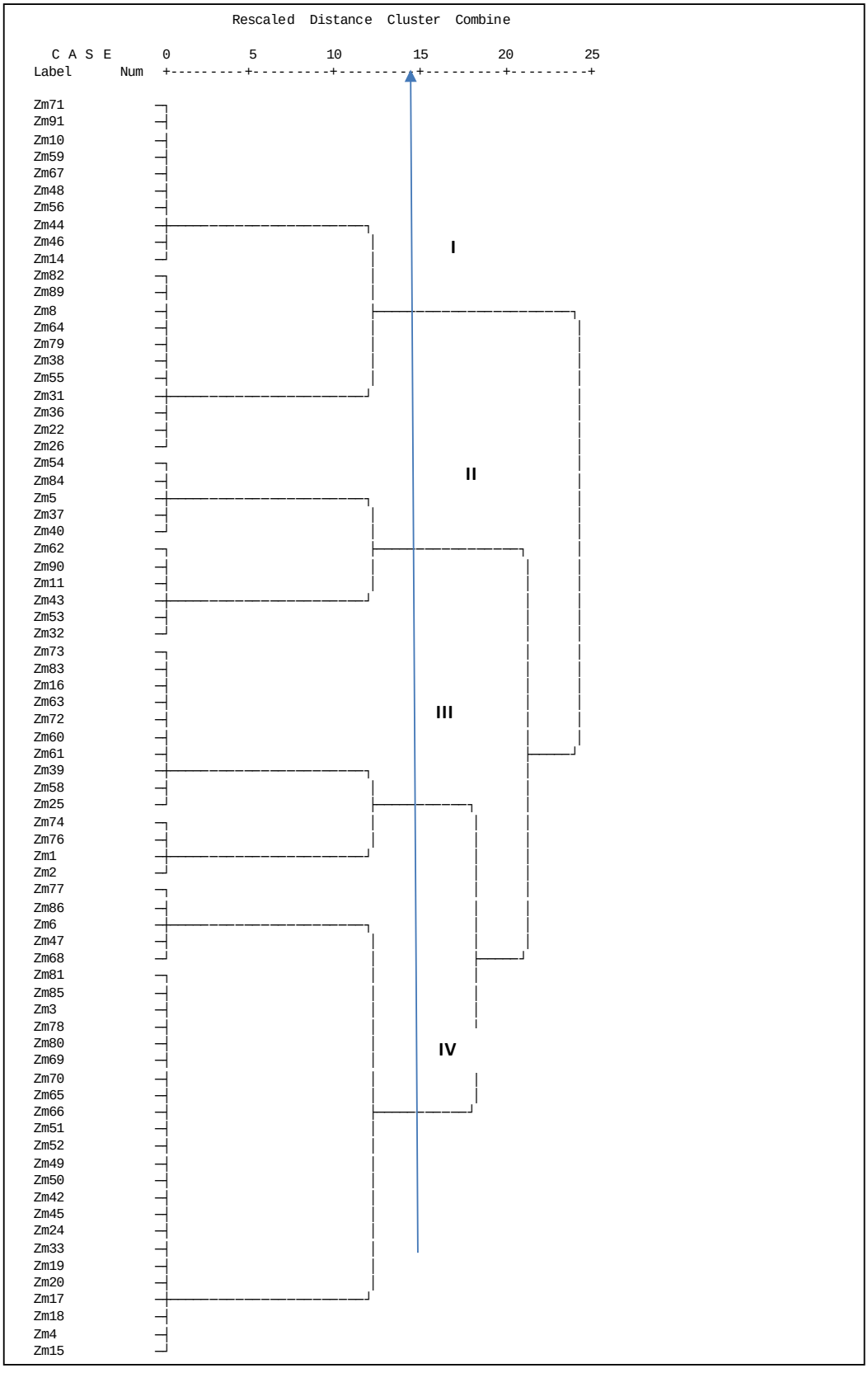

Figure 3. Dendrogram showing the genetic relationships between cultivars maize of South by UPGMA analysis.

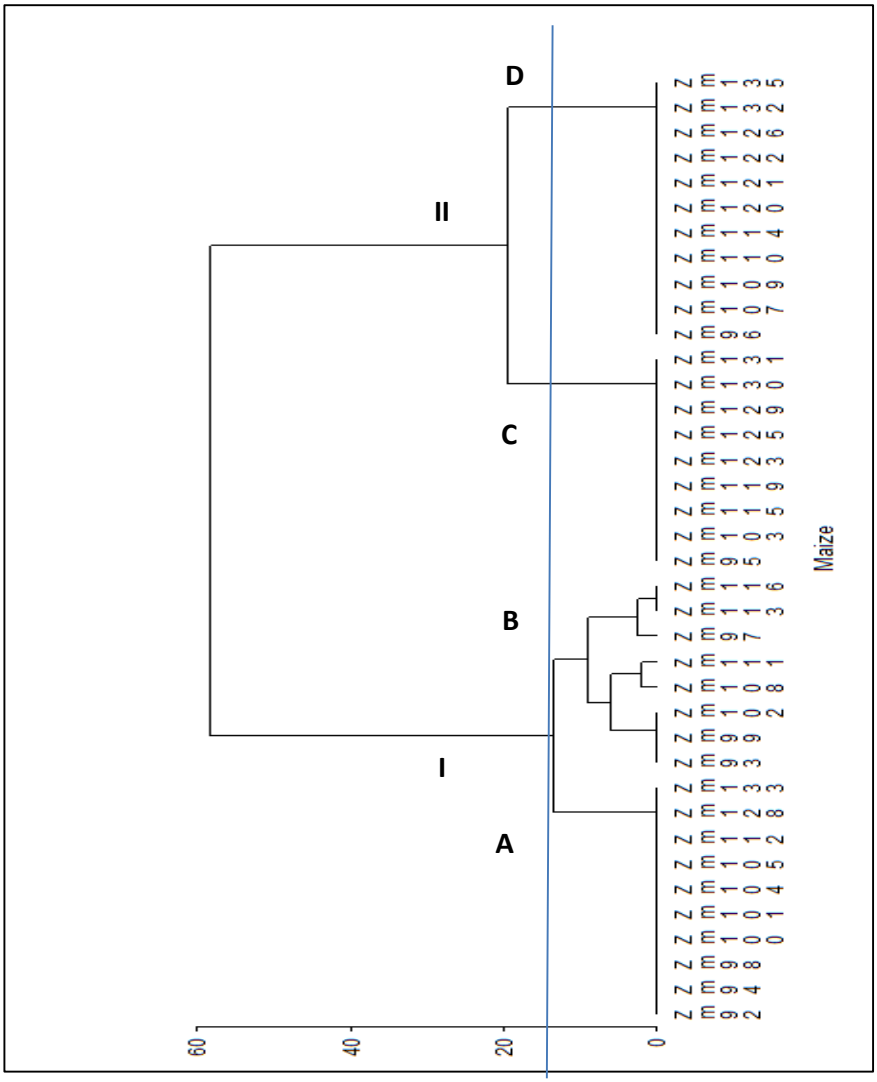

Figure 4. Dendrogram showing the genetic relationships between cultivars maize of Center by UPGMA analysis.

group shows a heterogeneous as I cluster and explained an eco-genetic relationship. The plants of this cluster are medium height and have bad husk cover. The cluster III is composed of 18 accessions and different from other clusters and the threshold of 0.90. The plants of this group were small height, have bad husk cover and have late germination time but early flowering. The cluster IV is different to the cluster III at the threshold of 0.10 and composed of 18 accessions. These plants are substantially similar to those of cluster III except the fact that these last cluster have average height and flowering (**Table 5**).

4. Discussion

4.1. Polymorphism Analysis

In this study, all microsatellite markers used were polymorphic and a high discriminatory power (0.71 average) that allowed discrimination of maize accessions from Benin by each marker. The high level of the PIC values showed that the fragments generated in this study were very informative. Al-Badeiry *et al.* [25] reported that the PIC demonstrates the informativeness of the SSR loci and their potential to detect differences among the varieties based on their genetic relationships. The efficiency of the molecular marker technique depends on the level of polymorphism and discriminatory power among the set of accessions [26]. The result obtained in this study

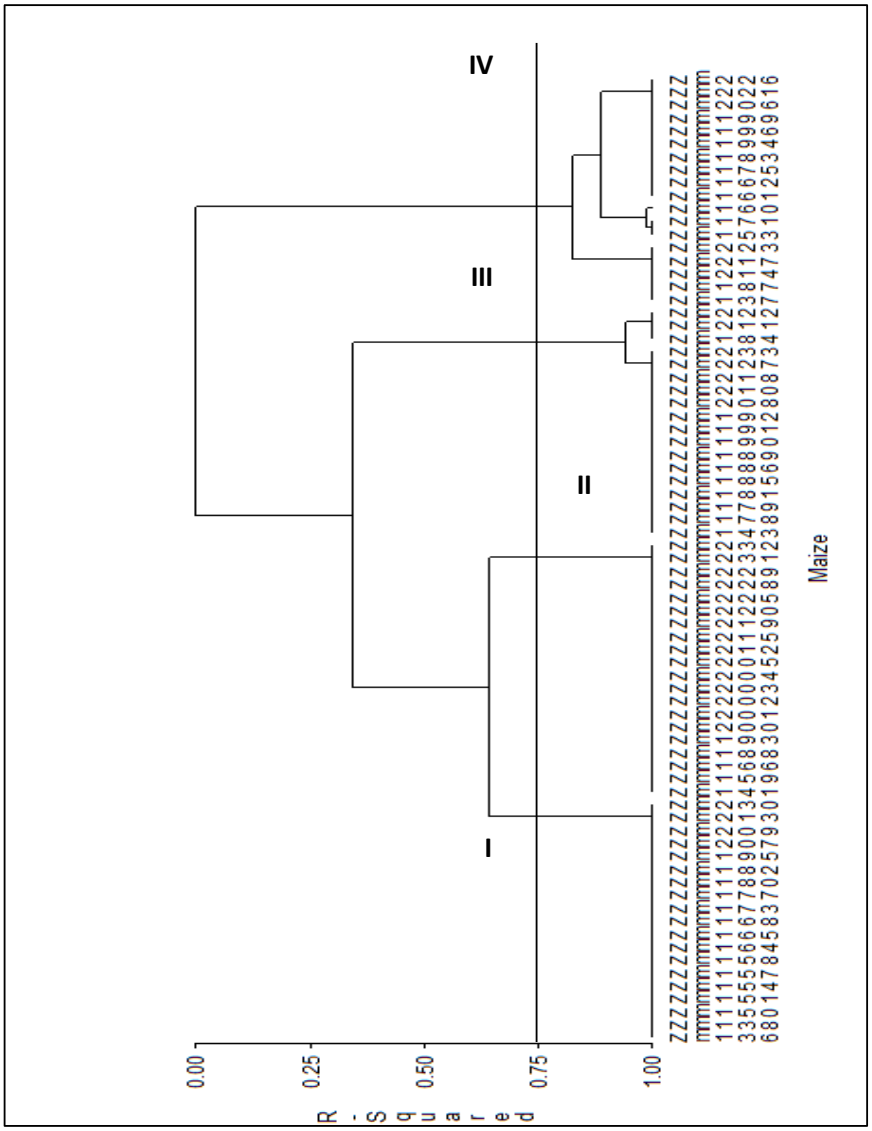

Figure 5. Dendrogram showing the genetic relationships between cultivars maize of North by UPGMA analysis.

were superior to that obtained by Shehata *et al.* [27] who obtained the PIC value of 0.57 on maize inbred lines in Saudi Arabia and also superior to the 0.44 funded by Al-Badeiry *et al.* [25] but similar to 0.69 obtained by Elçi and Hançer [28] on maize accession in Turkey. Considering the heterozygoty, the mean of 0.46 obtained in this study was lower than the one (0.54 and 0.55) obtained in previous studies [25] [29] on maize accession. However, our found is higher than those of Yao *et al.* [30] and Aci *et al.* [22], where they observed respectively an average value of 0.39 and 0.396.

4.2. Genetic Diversity of Maize Accession

In this study 227 distinct scored bands were recorded for Benin accessions. The Shannon index (0.52) obtained in this study seems high and may suggest a higher genetic diversity and differentiation of maize accession in Benin. These results were in agreement with the 0.54 Shannon index reported on sorghum using the microsatellites markers [31].

The higher diversity of maize accessions obtained in this study can be explained by the fact that during the collection of maize accession, several accessions (improve and local accession) were collected. In the different

agro-ecological zone, the farmers used to keep the accessions based not only in their culture but also in the nutritional characteristics. So because of their technological and organoleptic qualities found to be very different from local ecotypes, improved maize varieties developed by research are reported to be very few adopted and therefore little cultivated by peasants [32]. In addition, the cross-pollination between different varieties of maize from neighboring fields is also supplementary factors that increase genetic diversity. High levels of genetic diversity in maize are caused by active transposable elements, meiotic recombination following out crossing, new introgressions from exotic germplasm of this highly traded crop species, genetic drift following new introductions, and natural and artificial selection by farmers as the crop adapts to new environments [33].

To more understand the genetic diversity of maize accessions analyzed, the genotypic data obtained for three SSR markers were used to generate three UPGMA dendrograms depending on the area. Considering the dendrogram, a great similarity is observed between plants of the same group. However, the grouping of accessions in different cluster, reflects the genetic history, agronomic and eco-geographical affinity between the different accessions. The dendrograms revealed four different groups both in the north and in the south of Benin against two groups recorded among the center accessions. The highest diversity observed in the South and North can be explained by the fact that those areas are reported to be a large corn producing areas in Benin, incontrast with the Center area known to be producer of groundnuts and cassava [20].

Indeed, northern accessions were discriminated by the germinal parameters, plant and ear height, and early ears maturity while in the Center apart of the earliness and plant height the husk cover and sensitivity streak were considered. The discrimination of the accessions characteristics in the South is based on the germination time and female flowering [34]. This diversity of discriminative parameters depending on the area can not only be due to the difference of soil type but also to the climate. [35] asserted that farmers' choice of which maize genotype to grow is influenced by the major vegetation/climatic conditions found in Ghana. The traditional management of genetic resource of maize held by farmers participating in this great diversity of maize accessions.

5. Conclusion

In the present study, the SSR markers revealed the genetic relationships and diversity of maize accession in Benin. This study provides useful information that can be used in a breeding program for genetic improvement and characterization of new varieties. In addition, the results of this study are relevant for developing management the maize genetic resources. Further research on the sequencing of different maize gene is necessary to confirm the genetic diversity.

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Synapsin Polymorphisms Could Be Correlated with Stroop Simple Reaction Time Scores

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Abstract

Objective: The aim of this study was to research the relationship between Attention Deficit Hyperactivity Disorder (ADHD) and the synapsin III -196G>A and -631C>G polymorphisms and study their impact on neurocognition and behavior in Turkish children and adolescents. Methods: A total of 201 ADHD-diagnosed children and 100 control subjects aged between 8 and 15 years were recruited, and genetic material was obtained from saliva. In the diagnostic assessments, the K-SADS-PL semi-structured interview was applied. Children with any comorbid psychiatric diagnosis (with the exclusion of oppositional defiant disorder (ODD)), medical conditions, prior psychotropic drug use history or IQ score below 80 were excluded. For the behavioral and ADHD symptom assessments, the Turgay DSM-IV Disruptive Behaviors Rating Scale, Teacher Report Form (TRF) and Child Behavior Checklist (CBCL) were completed by the parents and teachers. Neurocognitive profiles were evaluated with the CNS-Vital Signs computerized neurocognitive test battery. Results: No significant difference in ADHD prevalence was observed between subjects with the synapsin III gene -196G>A polymorphism and -631C>G polymorphisms. These polymorphisms were also not associated with subtypes of ADHD. We found a relationship between both polymorphisms and

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Stroop simple reaction time. Conclusion: Synapsin's effect could be limited during childhood, but synapsin polymorphisms could be associated with Stroop simple reaction time.

Keywords

Attention Deficit Hyperactivity Disorder, Synapsin III, Association, Cognitive Function

1. Introduction

Attention-Deficit/Hyperactivity Disorder (ADHD) is a prevalent childhood neuropsychiatric condition, with an approximate worldwide prevalence of 5% in school-age children [1]. According to the current criteria of the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-V), ADHD is defined as a persistent pattern of inattention and/or hyperactivity-impulsivity that interferes with development [2]. Although ADHD maybe the most studied condition in child psychiatry worldwide [3], the precise causes and mechanisms are not yet completely understood. Genetics studies support a strong genetic contribution for ADHD development. The first genetic studies targeted dopaminergic genes, but they only explained a small part of ADHD heritability [4]. It is suggested that the modulation of neurotransmitter release and synaptogenesis might be involved in the pathophysiology of ADHD, as well [5].

Synapsins are a family of neuron-specific phosphoproteins [6] and synaptic vesicle proteins that play a very important role in the regulation of neurotransmission and synaptogenesis [7]. In mammals, three synapsin genes (I, II, and III) have been described [7]. Kao *et al*. cloned and characterized synapsin III from human DNA; it is located on chromosome 22 [8]. Synapsin III appears to play a significant role in early neurodevelopmental processes and also neurogenesis and axonogenesis [9]. Another important role of synapsin III is involved in synaptic transmission, especially dopamine release [10]. Studies have investigated genes encoding the proteins that have roles in the modulation of neurotransmitter release and ADHD, such as SNAP-25 gene (the synaptosomal-associated protein) [11], synaptobrevin-2 (VAMP2) and syntaxin 1A genes [12]. This inquiry about synapsin III in ADHD is commendable as ADHD is related to the release of neurotransmitters, especially dopamine.

The Synapsin III gene has been studied in schizophrenia and was mapped to chromosome 22 at 22q12-q13 [13]. This region is theorized to be involved in schizophrenia, but no relationship between synapsin III gene polymorphisms and schizophrenia [14] has been noted. Currently, there are two studies that have evaluated the relationship between Synapsin III and ADHD. One of the studies found no relationship between synapsin III gene polymorphisms and ADHD [15]. In the other study, Kenar *et al*. found a relationship between the -631C>G polymorphism and ADHD; however, they found no relationship between the -196G>A polymorphism and ADHD in adult Turkish patients [16].

The purpose of this study was to research the relationship between ADHD and the synapsin III gene and to investigate the neuropsychological tests in -196G>A and -631C>G polymorphisms in a clinical sample of children and adolescents diagnosed with ADHD, as well as in typically developing Turkish children and adolescents.

2. Methods

2.1. Study Design and Participants

In the present study, 201 ADHD-diagnosed children and 100 control subjects were evaluated, and the genetic material was assessed for the determination of synapsin III genotypes. The study participants were assessed in the child and adolescent psychiatry clinic of Ege University from December 2011 to March 2013.

The ADHD and control group subjects were recruited among children satisfying the following criteria: aged between 8 and 15 years, an IQ score above 80, living with his/her own family, attending a normal school, no history of head injury with unconsciousness, no history of neurological or other serious medical diseases or the constant use of prescribed medications for medical conditions, and no history of prior use of stimulants or use of psychotropic medication within the last six months. For the ADHD subjects, any psychiatric disorder other than

oppositional defiant disorder (ODD) was an exclusion criterion; for the control subjects, any psychiatric disorder was not allowed.

Ethical approval was granted and approved by the Pamukkale University Research Ethics Committee in accordance with the Helsinki Declaration. Written consent was taken from parents of the children both for the ADHD group and typical development (TD) group.

2.2. Diagnostic Procedures

The Child Behavior Checklist (CBCL) [17], the Teacher's Report Form (TRF) [18] and the DSM-IV Disruptive Behavior Disorders Rating Scale—teacher and parent forms [19] were completed by parents and teachers. Subjects with an inattentive score more than one standard deviation greater than the age norms for these scales were invited to participate in the diagnostic part of the study. This procedure was performed to guarantee that inattentive symptoms were adequately represented in all ADHD types assessed. Initially, a semi-structured interview (Kiddie-Schedule for Affective Disorders and Schizophrenia, present and life time version—K-SADS-PL) was administered to parents by a senior child psychiatry resident [20]. The same psychiatrist also performed a mental status examination of each child. Then, an estimated IQ was obtained by using vocabulary and block design subtests of WISC-R.

Two experienced child psychiatrists who were blind to the first diagnostic assessment conducted a confirmatory second diagnostic interview for those with a positive ADHD diagnosis in the first KSDAS-PL interview. The parents (mostly mothers—93%) and subjects were also interviewed according to the K-SADS-PL. "A best estimate procedure" was used to determine the final diagnoses [21]. Thirteen subjects were excluded from the second diagnostic part of the study. Three subjects refused to attend the second diagnostic interview, and a disagreement between interviewers on ADHD types/comorbid diagnoses occurred for 10 cases. Unrelated healthy controls were recruited from the same community. The same diagnostic procedure was applied for the assessment of controls. However, we required that TD subjects had inattentive scores one standard deviation below the mean for the child's age on the CBCL, TRF, ADHD-RS-IV scales. A sample of 201 children with ADHD and 100 control subjects were included in the study.

2.3. Neurocognitive Assessments: CNS Vital Signs

CNS Vital Signs is a computerized neurocognitive test battery that is composed of the following 7 tests: verbal and visual memory, finger tapping, symbol digit coding, the Stroop Test, a test of shifting attention, and the continuous performance test. The results are scored in the following 6 domains: neurocognitive index, memory, psychomotor speed, reaction time, comprehensive attention, and cognitive compliance. These scores are calculated according to the age group of the cases. This test is generally completed in 30 minutes. This battery is a sensitive tool for evaluating the performance of children and adolescents with ADHD [22].

2.4. Genotyping: DNA Isolation and Molecular Analysis

For the amplification of the synapsin III gene -196G>A (rs133945) polymorphism, DNA was isolated from peripheral blood leukocytes by the standard phenol/chloroform method and genotyped by the polymerase chain reaction-restriction fragment length polymorphism method. PCR was performed with a personal thermal cycler (Techgene), using SYN2 F-5'-T CCTTTCCAGAAGGATGTCC-3'/SYN2 R-5'AAGCCAACAAATACAT AAGTGGAGA-3'primers. For the amplification of the synapsin III gene -631C>G (rs133946) polymorphism, DNA was isolated from saliva. PCR was performed with a personal thermal cycler (Techgene), using SYN1 F-5'-AGGCATGTACTTGCGTTACC-3'/SYN1 R-5'-ACCAAATGACTACAAAGATGTACCA-3' primers.

2.5. Data Analyses

Group differences in the demographic variables were examined by the Kruskal-Wallis test for nonparametric data, and categorical comparisons were performed by Chi Square Test. Associations between the study groups and gene polymorphisms were examined using chi-squared analyses. Group comparisons in the scale and neurocognitive tests scores according to Synapsin III genotypes were performed with One-Way Analysis of Variance (One-Way ANOVA) for the parametric continuous data and with the Kruskal-Wallis Test for the nonparametric continuous data. Significant differences between pairs of groups were detected using Tukey's posthoc test for the parametric data and the Mann-Whitney U Test for the nonparametric data. p-values < 0.05 were con-

sidered statistically significant. SPSS (Statistical Package for Social Sciences) version 17.0 for Windows was used for statistical analysis of the data.

3. Results

A total of 201 ADHD patients and 100 healthy controls were admitted to the study. The mean age of the study group was 10.78 ± 2.01, and the mean age of the control group was 10.73 ± 1.92. There was no significant difference between the study and control groups regarding age ($p > 0.05$). The study group consisted of 78% boys and 22% girls. The control group consisted of 55% boys and 45% girls. The difference in sex composition between the groups was significant ($p > 0.001$) (**Table 1**).

3.1. Synapsin III Gene -196G>A Polymorphism Results of the Groups

Genomic DNAs for the synapsin III gene -196G>A polymorphism could not be obtained from 1 of the 201 patients and 1 of the 100 control subjects because of technical problems and they were not included into the results.

The frequency of the synapsin III gene -196G>A polymorphism was compared between groups. The G allele (patient: 51.3%, control: 47.5%) in the patient group, the A allele in the control group (patient: 48.7%, control 52.5%) ($p = 0.472$): and the G/A genotype (patient: 52.5%, control: 51.5%) ($p = 0.734$) were observed most often among the groups, and no significant difference was found between the groups. No significant difference was found between the groups in terms of the presence of the G ($p = 0.472$) or A allele ($p = 0.597$) (**Table 2**).

In comparing the ADHD subtypes and genotypes with the control group, the G/A genotype was most prevalent in all the three ADHD subtypes, in the inattentive type (44%), in the restrictive type (52%), in the combined type (57%) and also in the control group (51.5%). The difference between the subtypes and the control group was not significant ($p = 0.812$) (**Table 3**).

3.2. Neuropsychological Findings and Rating Scales of Synapsin III Gene -196G>A Polymorphism

We compared the CNS-VS domain scores according to the polymorphism groups in the entire sample; we found

Table 1. Age and sex distribution of the subjects according to diagnosis.

Group	Age (y)	p-value	Boysn (%)	Girlsn (%)	Totaln (%)	p-value
ADHD Controls	10.78 ± 2.01 10.73 ± 1.92	0.872*	157 (78%) 55 (55%)	44 (22%) 45 (45%)	201 (100%) 100 (100%)	**0.000****
Total	10.7 ± 0.98		212 (70.4%)	89 (29.6%)	301(100%)	

*Mann Whitney U nonparametric test. **Chi Square Test.

Table 2. Allele and genotype frequencies of the Synapsin III gene -196G>A polymorphism.

Allele frequencies	ADHDn (%)	Controln (%)	p-value
G A Total	205 195 400	94 104 198	0.472
Genotype frequencies	**n (%)**	**n (%)**	**p-value**
G/G G/A A/A Total	50 105 45 200	22 51 26 99	0.734
Presence or absence of G and A alleles	**n (%)**	**n (%)**	**p-value**
G/G + G/A	155 (77.5%)	73 (73.7%)	0.472
A/A + G/A	150 (75%)	77 (77.7%)	0.597

Chi-square test was performed. ADHD, attention deficit hyperactivity disorder.

statistical significance only for the Stroop simple reaction time (p = 0.008) (GG: 584.6 ± 373.1, GA: 451.6 ± 267.4, AA: 502.9 ± 269.8, respectively, post hoc analyses (Tukey); GG > GA; p = 0.005). The neuropsychological performance of the GG, GA and AA groups within the ADHD group did not differ significantly. We also could not find any difference between the GG + GA when compared to AA, or the GG when compared to GA + AA in the ADHD group.

DSM-IV Disruptive Behavior Disorders Rating Scale—teacher and parent forms and Child Behavior Checklist subgroups of the Synapsin III gene -196G>A polymorphism GG, GA and AA groups did not differ significantly. We did not find any difference in the DSM-IV Disruptive Behavior Disorders Rating Scale—teacher and parent forms or Child Behavior Checklist subgroups of the Synapsin III gene -196G>A polymorphism GG, GA and AA groups among the entire group. We also compared groups according to GG + GA and AA, as well as GG and GA + AA, but again did not find any significant difference.

3.3. Synapsin III Gene -631C>G Polymorphism Results of the Groups

Genomic DNAs for the synapsin III gene -631C>G polymorphism could not be obtained from 1 of the 201 patients and 1 of the 100 control subjects because of technical problems and they were not included into the results.

The groups were compared according to the Synapsin III gene -631C>G polymorphism. The C allele (patient: 50.5%, control: 48%): in the patient group, the G allele in the control group (patient: 49.5%, control 52%) (p = 0.562) and the C/G genotype (patient: 53.2%, control: 52.5%) (p = 0.886) were most prevalent among the groups, and no significant difference was found between the groups. No significant difference was found between the groups in terms of the presence of the C (p = 0.650) or G allele (p = 0.749) (**Table 4**).

In comparing the ADHD subtypes and genotypes with the control group, the C/G genotype was most prevalent in all the three ADHD subtypes, in the inattentive type (44%), in the restrictive type (50%), in the combined type (61%) and also in the control group (52.5%). The difference between the subtypes and the control group was not significant (p = 0.520) (**Table 5**).

Table 3. Synapsin III gene -196G>A polymorphism genotype distribution of the ADHD subtypes and the control groups.

Genotype	Inattentive type n (%)	Restrictive type n (%)	Combined type n (%)	Control	p-value
GG	15 (30%)	12 (24%)	23 (23%)	22 (22.2%)	0.812
GA	22 (44%)	26 (52%)	57 (57%)	51 (51.5%)	
AA	13 (26%)	12 (24%)	20 (20%)	26 (26.3%)	
Total	50	50	100	99	

Chi-square test was performed.

Table 4. Allele and genotype frequencies of the Synapsin III gene -631C>G polymorphism.

Allele frequencies	ADHD n (%)	Control n (%)	p-value
G	203	95	0.562
A	199	103	
Total	402	198	
Genotype frequencies	**n (%)**	**n (%)**	**p-value**
C/C	48	22	0.886
C/G	107 (53.2%)	52 (52.5%)	
G/G	46	25	
Total	201	99	
Presence or absence of C and G alleles	**n (%)**	**n (%)**	**p-value**
C/C + C/G	155 (77.1%)	74 (74.7%)	0.650
G/G + C/G	153 (76.1%)	77 (77.7%)	0.749

Chi-square test was performed. ADHD, attention deficit hyperactivity disorder.

Table 5. Synapsin III gene -631C>G polymorphism genotype distribution of the ADHD subtypes and the control groups.

Genotype	Inattentive type n (%)	Restrictive type n (%)	Combined type n (%)	Control	p-value
CC	15 (30%)	12 (24%)	21 (21%)	22 (22.2%)	0.520
CG	21 (44%)	25 (50%)	61 (61%)	52 (52.5%)	
GG	14 (26%)	13 (26%)	19 (19%)	25 (25.3%)	
Total	50	50	100	99	

Chi-square test was performed.

3.4. Neuropsychological Findings and Rating Scales of Synapsin III Gene -631C>G Polymorphism

We compared the CNS-VS domain scores of the CC, CG and GG groups in the entire sample, and we found a significant difference in the Stroop simple reaction time ($p = 0.002$) (CC: 598.4 ± 374.6, CG: 448.2 ± 259.1, GG: 499.9 ± 300.3, respectively, post hoc analyses (Tukey); CC > CG; $p = 0.001$). In comparing the CC, CG and GG groups, the only significant difference was in the Stroop simple reaction time ($p = 0.022$) (CC: 598.0 ± 373.2, CG: 458.8 ± 248.1, GG: 536.7 ± 310.3, respectively) in the ADHD group. We did not find any difference between the CC + CG and GG groups in neuropsychological performance. We again found a difference in Stroop simple reaction time in comparing the Synapsin III gene -631C>G polymorphism CC and CG + GG groups ($p = 0.020$) (CC: 598.0 ± 373.2, CG + GG: 48227 ± 269.6, respectively).

DSM-IV Disruptive Behavior Disorders Rating Scale – teacher and parent forms and Child Behavior Checklist subgroups of the Synapsin III gene -631C>G polymorphism CC, CG and GG groups did not differ significantly. We did not find any difference in the DSM-IV Disruptive Behavior Disorders Rating Scale—teacher and parent forms and Child Behavior Checklist subgroups of the Synapsin III gene -631C>G polymorphism CC, CG and GG groups in the entire group. We also compared the CC+CG and GG groups and the CC and CG + GG groups, but again, we did not find any significant difference.

4. Discussion

In the present study, we did not observe a relationship between ADHD and the -196G>A polymorphism of the synapsin III gene. These findings are compatible with the literature. Makkar *et al*. investigated the relationships of five polymorphisms of the synapsin III gene; rs133946 (exon 5), rs133945 (exon 5), rs242089 (intron 5), rs3788459 (intron 6) and rs1056484 (3'UTR) with ADHD in 177 families with 220 affected children. They found no relationship between the -196G>A polymorphism and ADHD [15]. In the study with a total of 139 patients with adult ADHD and 106 healthy controls, no significant difference was observed between the groups in the prevalence of the synapsin III gene -196G>A polymorphism [16]. This polymorphism may not have an effect on ADHD symptoms.

We did not find a relationship between ADHD and the -631C>G polymorphism of the synapsin III gene. The literature on ADHD and the -631C>G polymorphism is controversial. Makkar *et al*. found no relationship in children [15]. However, Kenar *et al*. found a significant relationship between ADHD and the -631C>G polymorphism of the synapsin III gene in adult patients [16]. The difference between the aforementioned adult study and ours may be because their patient and control groups consisted of adults and our groups consisted of children and adolescents. Additionally, this polymorphism may not have a major effect on ADHD symptoms during childhood but may present at adulthood. It is indicated that synapsin III plays a much earlier role in neurodevelopment [9], but interestingly, we did not find a relationship with ADHD in childhood.

There have been no data on neuropsychological performance and the -196G>A polymorphism and -631C>G polymorphism until now. In the entire group, for the -196G>A polymorphism, the GG group had higher Stroop test scores than the other two groups and significantly higher scores than the GA group. Also in the entire group, for the -631C>G polymorphism, the CC group's Stroop test scores were higher than the other groups'. There are several versions of the Stroop test, and the modification adopted for CNS Vital Signs uses only four colors/color words (red, green, yellow, blue), and only one key, the space bar. The test has three parts. In the first section, the words "red", "yellow", "blue" and "green" appear at random on the screen, and the subject presses the space bar

as soon as he or she sees the word, and then, the subject receives a simple reaction time score [22]. In one study of 175 patients with ADHD, patients aged 10 to 29 were compared with 175 age-matched normal controls who had taken the CNS Vital Signs battery. The ADHD group had higher Stroop test simple reaction time scores than the normal controls. A lower score on the Stroop test is considered better [23]. In both polymorphism groups, we observed an effect on Stroop simple reaction time scores. We did not find any study on ADHD, but it was shown that synapsin III protein levels significantly decreased in the prefrontal cortex of individuals with schizophrenia [24]. Additionally, hemodynamic brain imaging data both support the sensitivity and specificity of the Stroop test to indicate frontal lobe functioning [25]. Accordingly, these polymorphisms may have affected the Stroop test simple reaction time in our study via this mechanism.

4.1. Limitations

There are several limitations of the present study. Our study included only two ADHD subtypes (ADHD-I and ADHD-C) because ADHD-H is rarely observed clinically. Future studies that include the ADHD-H subtype would provide more comprehensive information related to the psychopathology of ADHD. We did not exclude ODD, and the presence of possible comorbid ODD in some patients may have had a confounding effect. Additionally, we assessed only estimated IQ using the vocabulary and block design subtests of the WISC-R. Thus, other potential ADHD neuropsychological domains were not assessed (e.g., delay aversion, RT variability). For the DNA source, we used a saliva sample, but peripheral blood samples could be taken to increase the efficiency of genotyping.

4.2. Conclusion

Attention Deficit Hyperactivity Disorder is complex and heterogeneous. It is difficult to find a particular gene associated with ADHD. Prospective studies, which followed subjects with ADHD from child- to adulthood, have shown a reduction in hyperactive and impulsive symptoms over time, but the inattentive symptoms persisted into adulthood [26]. Therefore, the expression of the main genes could be different in childhood than in adulthood. Synapsin effects could be limited during childhood but may be present in adulthood. Therefore, specific endophenotypes for attention deficit, hyperactivity and impulsivity symptoms should be determined.

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Phosphorylation on TRPV4 Serine Residue 824 Enhances Its Association with PGM1

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Abstract

The TRPV4 cation channel is expressed in a broad range of tissues and participates in the generation of a Ca^{2+} signal and/or depolarization of membrane potential. Here, human phosphoglucomutase-1 (PGM1), an enzyme that converts glucose-6 phosphate to glucose-1 phosphate in the glycolysis pathway, as the first auxiliary protein of TRPV4 Ca^{2+} channels, is identified with yeast two hybrid system, coimmunoprecipitation, confocal microscopy, and GST pull-down assays. TRPV4 forms a complex with PGM1 through its C-terminal cytoplasmic domain. Because it is demonstrated that TRPV4 serine residue 824 (S824) is phosphorylated by serum/glucocorticoid regulated kinase 1, we elucidate the effect of TRPV4 S824 phosphorylation on TRPV association with PGM1. Even an inactivated mutant version of TRPV4, S824A, exhibited a decreased ability to bind PGM1, an activated phosphomimetic mutant version of TRPV4, S824D, exhibited enhanced binding to PGM1. Thus, formation of the TRPV4/PGM1 complex and localization of this complex to the plasma membrane appear to be regulated by the phosphorylation status of residue S824 in TRPV4. The newly identified interactor of TRPV4 may help the molecular pathways modulating transport activity or glucose metabolism, respectively.

Keywords

Membrane Localization, Phosphoglucomutase1, Phosphorylation, Protein-Protein Interaction, TRPV4

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1. Introduction

The ubiquitously expressed TRPV4 cation channel, a member of the TRP vanilloid subfamily, generates a Ca^{2+} signal and/or depolarizes the membrane potential (see reviews [1]-[4]). Through its contributions to osmo-and mechanotransduction, TRPV4 plays a role in cellular and systemic volume homeostasis, arterial dilation, nociception, epithelial hydroelectrolyte transport, bladder voiding, and regulation of ciliary beat frequency [5]-[10]. TRPV4 also responds to temperature, endogenous arachidonic acid metabolites, and phorbol esters, including the inactive 4α-phorbol 12, 13-didecanoate [11] [12]. In addition, TRPV4 also participates in receptor-operated Ca^{2+} entry; thus, showing multiple modes of activation [1]-[4]. In this sense, several proteins have been proposed to modulate TRPV4 subcellular localization and/or function, including microtubule-associated protein 7, calmodulin (CaM), PACSIN3, and no lysine protein kinase [13]-[16]. Furthermore, a close functional and physical interaction has been described between the inositol triphosphate receptor 3 (IP3R3) and TRPV4; this interaction was shown recently to sensitize the latter to the mechano- and osmotransducing messenger, 5′-6′-epoxieicosatrienoic acid [17] [18].

Glucose 1-phosphate is not a useful metabolic intermediate; however, phosphoglucomutase1 (PGM1) catalyzes conversion of glucose 1-phosphate to glucose 6-phosphate [19]. After glycogen phosphorylase catalyzes the phosphorolytic cleavage of a glucosyl residue from the glycogen polymer, the liberated glucose molecule retains a phosphate group at the 1-carbon position [20]. Because glucose 1-phosphate molecule is not a useful metabolic intermediate, PGM1 catalyzes further the conversion of glucose 1-phosphate to glucose 6-phosphate [21]. The metabolic fate of newly generated glucose 6-phosphate depends on the energy requirements of the cell at the time it is generated. If the cell is in a low-energy state, glucose 6-phosphate will be metabolized along the glycolytic pathway, eventually yielding two molecules of adenosine triphosphate [22] [23]. On the other hand, the cell is in need of biosynthetic intermediates, glucose 6-phosphate enters the pentose phosphate pathway, where it undergoes a series of reactions to yield ribose and/or NADPH, depending on cellular conditions [24].

In the liver, glucose 6-phosphatase can also catalyze conversion of glucose 6-phosphate to glucose, which exits the liver and is transported to other cells. However, muscle cells lack glucose 6-phosphatase, so they cannot share their glycogen stores with the rest of the body [23]. PGM1 acts in the opposite fashion when blood glucose levels are high. In this case, PGM1 catalyzes the conversion of glucose 6-phosphate, a product generated from glucose by hexokinase, to glucose 1-phosphate. Glucose-1-phosphate then reacts with UTP to yield UDP-glucose in a reaction catalyzed by UDP-glucose-pyrophosphorylase [23] [24]. If activated by insulin, glycogen synthase will attach the glucose molecule from the UDP-glucose complex onto a glycogen polymer [23]. In yeast, PGM1 activity results in elevated Ca^{2+} in a PGM2 mutant grown in medium containing Gal as the carbon source. In that study, either Mg^{2+} or Ca^{2+} ions were required for PGM1 activity [25].

The aim of the present study was to identify auxiliary proteins that specifically interact with TRPV4 [26]. To this end, we performed a putative motif searching screen to identify proteins associated with epithelial Ca^{2+} channels, and identified PGM1 as a candidate protein. Through validation studies, we found that the interaction between TRPV4 and PGM1 was enhanced by phosphorylation of TRPV4 serine residue 824 (S824). Functional interaction between TRPV4 and PGM1 was further substantiated by pull-down assays, immunohistological studies, and a Ca^{2+} ion imaging analysis, as TRPV4 subcellular localization and activity were shown to depend on PGM1. We propose that PGM1 preferentially associates with phosphorylated TRPV4 (the activated channel form); however, binding of PGM1 with TRPV4 negatively impacts its Ca^{2+} channel activity.

2. Materials and Methods

2.1. Site-Directed Mutagenesis

To generate phosphomimetic and other mutants, amino acid changes were introduced using the appropriate oligonucleotides for S/A (forward, 5′-agg gat cgttgg**GccGcg**gtggtgccccgc gta-3′; reverse, 5′-gcggggcaccac**cgCggC** ccaacgatccct acg-3′) or S/D (forward, 5′-agg gat cgttgg**GAc GAC** gtggtgccccgc gta-3′; reverse, 5′-gcggggcaccac **GTC gTC**ccaacgatccct acg-3′) and wild-type TRPV4 cDNA as a template. TRPV4 mutant constructs were prepared using a QuickChange® Multi Site-Directed Mutagenesis Kit (Stratagene). To generate the construct expressing a truncated version of TRPV4 (Δ718-871), the following primers were used: forward, 5′-ataggatcc-atgggt gag accgtgggc cag-3′; reverse, 5′-atactc gag cta cag tggggcatcgtc cgt-3′. All TRPV4 mutants were confirmed by DNA sequencing. Human embryonic kidney (HEK293) cells were transfected with TRPV4 and with the appropriate mutant constructs, as described previously.

2.2. Glutathione S-Transferase (GST)-TRPV4 Fusion Proteins and Pull-Down Assays

TRPV4 sequences were PCR-amplified, subcloned into pGEX-5X-1, sequenced, and expressed in *Escherichia coli* BL21 cells. S824A-agarose or GST-TRPV4 fusion proteins bound to glutathione-Sepharose resin were equilibrated in PBS buffer containing 0.1% Triton X-100 and either 1 mM $CaCl_2$ or 2 mM EGTA. Incubation of immobilized fusion proteins with total cell lysates or recombinantly purified S824A fusion protein was followed by three washes with the appropriate buffers. Bound proteins were eluted with sample buffer, resolved by SDS-PAGE, and detected by chemiluminescence through exposure to X-ray film (Fuji Las 3000 mini).

2.3. Fluorescence Measurements of $[Ca^{2+}]_i$

Measurements of $[Ca^{2+}]_i$ were obtained using the fluorescent Ca^{2+} indicator, Fluo4-acetoxymethyl ester (Fluo4-AM), as previously described. In brief, cells grown on cover slips were incubated in the dark for 40 min in DMSO solution containing 1 μM Fluo4-AM at 24˚C, and then washed and incubated for 15 min to allow hydrolysis of internalized Fluo4-AM. Measurements of $[Ca^{2+}]_i$ in single cells were then obtained by examining the emitted fluorescence on a confocal microscope (LSM710 Zeiss, Germany) at wavelengths 495 nm (excitation) and 519 nm (emission). Absorption values (in arbitrary units) obtained with the argon-ion laser at 488 nm are expressed as relative intracellular Ca^{2+} ion concentrations $[Ca^{2+}]_i$. All experiments were carried out at 24˚C. After stimulation with mild heat (from 24˚C to 42˚C within 45 s for 2 min), $[Ca^{2+}]_i$ was measured in single cells at 24˚C.

2.4. Confocal Microscopy

MDCK or HEK293 cells were seeded overnight at 60% confluence onto culture slides coated with human fibronectin (Becton Dickinson). Cells were washed several times with ice-cold PBS and then fixed in 3% paraformaldehyde for 10 min. Fixed cells were permeabilized with 0.1% Triton X-100 for 10 minutes and then blocked for 1 h in PBS containing 5% BSA (Sigma, USA) and 0.1% Tween. After an additional 20 minutes of incubation at 37˚C, cells were fixed, permeabilized, and immunostained with either anti-PGM1 or anti-TRPV4 antibodies. Cells were then incubated with secondary Alexa Fluor 568-conjugated donkey anti-rabbit or Alexa Fluor 488-conjugated goat anti-mouse antibodies (Molecular Probes, Inc., Eugene, OR, USA). Confocal microscopy analysis was performed with an LSM710 microscope (Zeiss, Germany) at the Center for Experimental Research Facilities at Chungbuk National University.

2.5. Plasmids and Protocol for Yeast Two-Hybrid Screening

The sequence encoding TRPV4 was subcloned into the bait plasmid (PCR primers: forward, 5'-CCCCAT ATGATGGGTGAGACCGTGGGC-3'; reverse, 5'-GGGGATCCGCAGTGGGGCATCGTCCGT-3'). The resultant plasmid was designated TRPV4-pGBKT7. A human liver cDNA library (Clontech) was screened for proteins that interacted with TRPV4 using the Matchmaker Gold Yeast Two-Hybrid System (Clontech protocol PT4084-1). The bait plasmid, TRPV4-pGBKT7, was transformed into the AH109 yeast strain; around 2×10^3 transformants were screened. Transformants were assayed for MEL1 activation by selection on high-stringency medium plates: SD/-Ade/-His/-Leu/-Trp/X-α-gal. Prey plasmids were recovered from positive yeast clones and retransformed into DH5α cells. Transformants harboring AD plasmids were selected on LB plates with 100 μg/ml ampicillin, and plasmid inserts were identified by DNA sequencing.

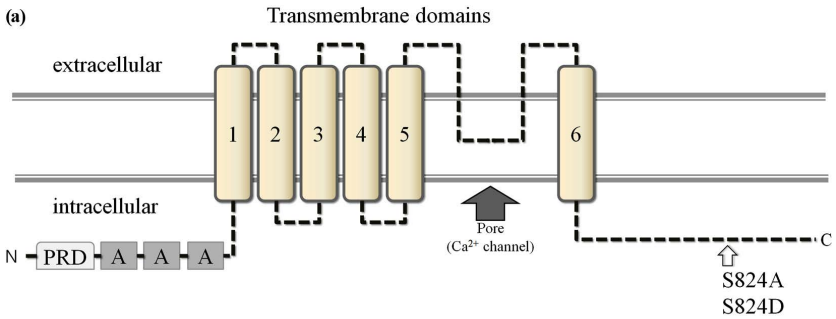
2.6. Solutions and Drugs

Cells were normally superfused with a solution containing (in mM): 88 NaCl, 5 KCl, 5.5 glucose, 1 $CaCl_2$, 10 HEPES and 100 mannitol, adjusted to pH 7.4 with NaOH (300 mosm kg^{-1} H_2O). The HTS was adjusted to 200 mosm kg^{-1} H_2O by omitting mannitol. 4-αPDD was purchased from Sigma (St. Louis, MO, USA). Fluo-4AM and Fura-2AM were acquired from Molecular Probes, Inc. Stock solutions of phorbol esters were initially prepared in dimethyl sulphoxide (DMSO) at a concentration of 1 mM, and then stored at −20˚C. The final DMSO concentration in the experimental bath solution containing phorbol esters never exceeded 0.5%. Insulin and GSK650394 (an SGK1 inhibitor) were acquired from Tocris Bioscience (Ellisville, MO, USA), and used according to the manufacturer's recommendations.

3. Results

3.1. Identification of PGM1 as a TRPV4-Associated Protein-Protein Interaction

To identify proteins that interact with TRPV4, the C-terminal tail of TRPV4 (**Figure 1(a)**) was used to screen a mouse kidney cDNA library using a yeast two hybrid assay. One of the positive clones encoded PGM1, which catalyzes to convert glucose 6-phosphate (which is easily generated from glucose by the action of hexokinase) to glucose 1-phosphate. PGM1 strongly interacted with TRPV4, whereas no binding was observed with ENaC, indicating specificity of the PGM1-TRPV4 interaction. The subunit of the epithelial Na^+ channel, ENaC, was used as a negative control. In addition β-galactosidase activity was not detectable in the absence of prey, or after co-transformation of the bait with the empty pACT2 (prey) vector (data not shown). The serine residue 824 in the C-terminal domain is indicated. Mutants of S824 (in ^{811}shtvgrlr**R**d**R**ws**SV**vprvvel^{832}), which was constructed for this study, are also detailed in **Figure 1(b)**. EGFP (or GST)-tagged mouse TRPV4 WT, S824A (S824 nonphosphorylatable mutant), or S824D (S824 phospho-mimicking mutant) fusion proteins were expressed in the HEK293 cell line, purified with EGFP monoclonal antibody (or Glutathione beads), and utilized as antigen proteins.



(b)

^{811}shtvgrlr**R**d**R**ws**S**V**V**prvvel^{832} WT

(RXRXX(S/T)Φ; where Φ is any hydrophobic amino acid)

^{811}shtvgrlr**R**d**R**ws**AV**vprvvel^{832} S824A

^{811}shtvgrlr**R**d**R**ws**DV**vprvvel^{832} S824D

(c)

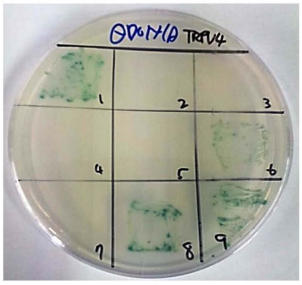


Figure 1. Predicted topology of the TRPV4 C-terminal domain and yeast two-hybrid system. (a) Transmembrane topology of mouse TRPV4 (871 residue). The three ankyrin binding repeats (ANK, gray circles), the six transmembrane regions (TM1 - TM6), the PGM1 binding site (PGM1), and the putative SGK1 phosphorylation site (S824) are indicated. The C-terminal cytoplasmic region of TRPV4 (Δ718-871) is also indicated. The putative SGK1 phosphorylation site (S824) is present in the PGM1 domain (residues 812 - 832) of the TRPV4 channel. The line indicates the GST C-terminal cytoplasmic region of TRPV4 (Δ718-871) or the mutant (S824A) fusion protein; the TRPV4 mutant site (S824A or S824D) is compared with the wild-type (WT, Gene Bank no. BC127052). (b) Alignment of TRPV4 WT, S824A, and S824D mutant versions with the consensus SGK1 substrate motif. The putative SGK1 phosphorylation site (S824) of TRPV4 is located in the specific conserved SGK1 substrate region [R-X-R-X-X-(S/T) Φ]. The S824A and S824D mutant versions were constructed using site-directed mutagenesis. (c) Screening of TRPV4-interacting proteins by the yeast two-hybrid system. Yeast were transformed with a plasmid driving expression of TRPV4, and positive colonies were selected with leucine, tryptophan, adenine, histidine, and aureobasidin A. One, six, eight and nine are positive clones from the first round of selection from a human liver library using TRPV4-pGBKT7 as bait. All four clones contained the same gene (human PGM1), as revealed by DNA sequencing.

Homo sapiens phosphoglucomutase 1 (PGM1) was obtained from a human liver library (Clontech, Palo Alto, CA, USA) by polymerase chain reaction with the primers 5′-TAATACGACTCACTATAGGG-3′ and cloned into the pEGFP-C2 vector (Clontech) to generate the PGM1-pEGFP-C2plasmid. The entire coding region of the PGM1 was amplified and subcloned into the pGEX-5X-1 vector to construct the GST fusion protein.

3.2. Interaction between PGM1 and the C-Terminal Domain of TRPV4

GST pull-down binding assays were performed to further establish the interaction between TRPV4 and PGM1 (**Figure 2(a)**). The endogenous PGM1-containing MDCK homogenate was incubated with GST-TRPV4 or GST-TRPV4 (Δ718-871) fusion proteins immobilized on glutathione-Sepharose 4B beads. PGM1 bound specifically to the C-terminal tail of TRPV4, as no interaction was observed with GST-TRPV4 (Δ718-871) (**Figure 2(b)** left lane). Thus, the C-terminal tail of TRPV4 is required for the interaction with STM1. Taken together, our observations suggest that TRPV4 interacts directly with PGM1 through the C-terminal domain of TRPV4 in MDCK cells.

3.3. Phosphorylation of TRPV4 S824 Enhances the Interaction between TRPV4 and PGM1

We demonstrated previously that the interaction between TRPV4 and actin (STIM1 or tubulin) is differentially regulated by phosphorylation of TRPV4 S824 (**Figure 1(b)**; [27]). Similarly, we hypothesized that phosphorylation of TRPV4 on S824 regulates the interaction between TRPV4 and PGM1. Co-immunoprecipitation and GST pull-down assays of these proteins in their native forms were performed to investigate the consequences of TRPV4 S824 phosphorylation on the interaction between TRPV4 and PGM1 (**Figure 3**). HEK 293 cells, which do not express endogenous TRPV4, were transiently transfected with plasmids driving expression of either EGFP-TRPV4 WT or S824A. After 48 h, cell lysates were generated, and immunoprecipitation was conducted

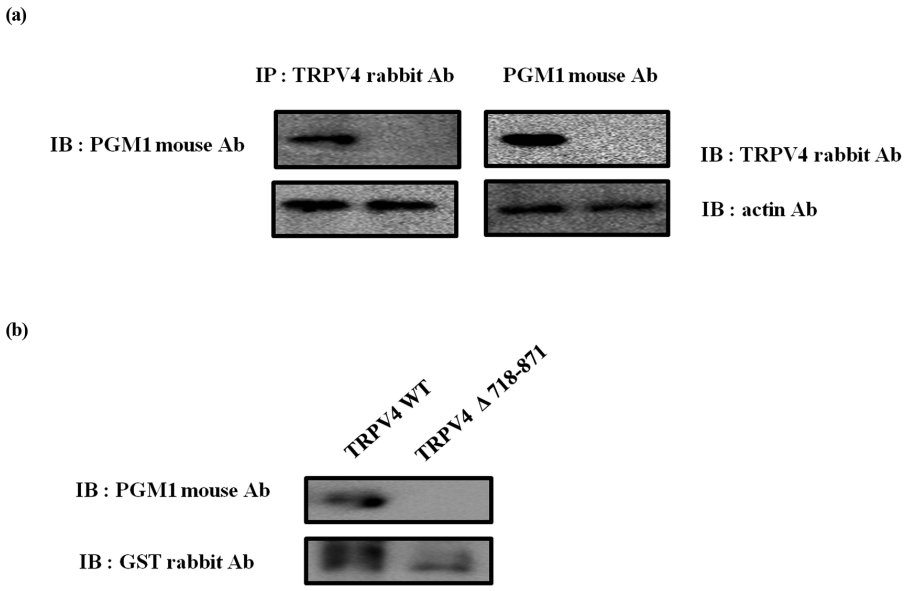


Figure 2. Protein-protein interaction between PGM1 and the C-terminal domain of TRPV4. (a) Interaction between TRPV4 and PGM1 in MDCK cells. An immunoblot (IB) analysis was performed using antibodies against PGM1 (left) following immunoprecipitation (IP) using anti-TRPV4 antibodies. Conversely, anti-PGM1-immunoprecipitated complexes were subjected to immunoblot analysis using anti-TRPV4 antibodies (right). Co-immunoprecipitation of PGM1 with TRPV4 confirmed the presence of the TRPV4-PGM1 complex. An unrelated antibody was used as a negative control for immunoprecipitation. Antibodies against actin were used as a control for immunoblot analysis (bottom). (b) The C-terminal domain of TRPV4 is required for its interaction with PGM1. After incubating GST-TRPV4 WT or a deletion mutant fusion protein (Δ718-871) with a HEK293 cell lysate, proteins bound to purified GST beads were detected by immunoblotting with antibodies against PGM1 (upper lane). The deletion mutant lacking the C-terminal region (Δ718-871) did not pull down PGM1 (right lane), whereas GST TRPV4 WT did (left lane).

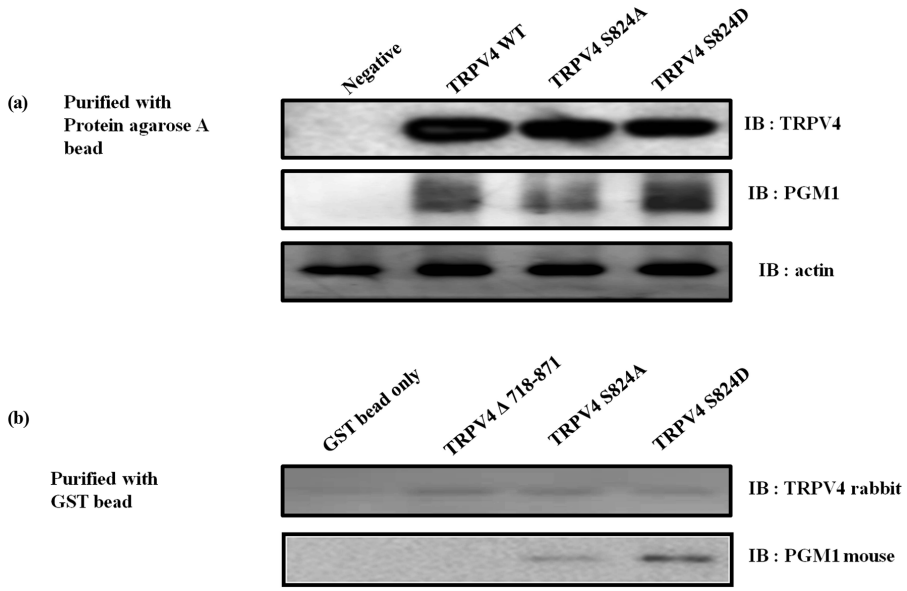


Figure 3. Effects of phosphorylation of TRPV4 S824 on the interaction between TRPV4 and PGM1. (a) Co-immunoprecipitation of TRPV4 WT, S824A, or S824D mutant versions with PGM1. HEK 293 cells were transiently transfected with plasmids driving expression of EGFP-TRPV4 WT or S824A. Cell lysates were generated after 48 h, and pull-down assays from total protein extracts were performed with protein A-agarose beads. Western blot assays were performed with rabbit anti-TRPV4 or anti-PGM1 antibodies. (b) Pull-down analysis of PGM1 with GST-fusion C-terminal domains of TRPV4 WT, S824A, or S824D. GST-fusion proteins encompassing C-terminal TRPV4 domains were constructed and expressed in *E. coli*. Approximately 2 µg of WT, S824A, or S824D fusion protein bound to glutathione-Sepharose beads was incubated with HEK 293 cell lysates.

with mouse anti-TRPV4 or anti-PGM1 antibodies. Immunoblotting was then performed with rabbit anti-TRPV4 or anti-PGM1 antibodies (**Figure 3(a)**). Interestingly, TRPV4 S824A (a mutant mimicking unphosphorylated TRPV4) did not co-precipitate PGM1 (**Figure 3(a)**, middle lane), whereas TRPV4 S824D (a phosphomimetic mutant form of TRPV4) and TRPV WT did. These results indicate that the phosphomimetic version of TRPV4 (S824D) exhibited a higher affinity for PGM1 than a version mimicking the unphosphorylated protein (S824A).

We performed pull-down assays of PGM1 using GST-fusion proteins of the C-terminal domain of TRPV4 WT, S824A, or S824D to further test the hypothesis that the interaction between TRPV4 and PGM1 is regulated by the phosphorylation status of TRPV4 S824 (**Figure 3(b)**). GST-fusion proteins encompassing C-terminal TRPV4 domains were constructed and expressed in *E. coli*. Approximately 2 µg of WT, S824A, or S824D fusion protein immobilized on glutathione-Sepharose resin was incubated with HEK 293 cell lysates. Similar to the results shown in **Figure 3(a)**, PGM1 was captured from HEK 293 cell lysates by the GST-tagged C-terminal portion of TRPV4 when it was immobilized on glutathione-Sepharose 4B beads; thus, demonstrating a physical interaction between TRPV4 and PGM1 (**Figure 3(b)**). A greater amount of PGM1 was captured by TRPV4 S824A compared with TRPV4 WT, a result consistent with the data shown in **Figure 2(a)**. Taken together, these data suggest that PGM1 preferentially binds TRPV4 when S824 is phosphorylated, and led us to hypothesize that the function of this interaction is to inhibit its channel activity (**Figure 6**).

3.4. Effects of PGM1 on Phosphorylated TRPV4 S824 and TRPV Binding with PGM1

As a complementary approach to examine whether phosphorylation of TRPV4 S824 enhances its binding with PGM1, HEK 293 cells were transiently cotransfected with EGFP-TRPV4 WT, S824A, or S824D, and pmCherry-PGM1 (pmCherry-N1, Clontech) expression plasmids. After 48 h, the cells were fixed, immunostained, and examined by confocal microscopy. Ectopically-expressed TRPV4 (green) and PGM1 (red) were colocalized in the cytosol (yellow) (**Figure 4**). As shown in **Figure 4** (upper lane), we observed that ectopically-expressed TRPV4 WT was in close proximity to PGM1 in HEK293 cells. However, TRPV4 S824A did not colocalize with PGM1 (middle lane), whereas TRPV4 S824D colocalized with PGM1 (bottom lane), consistent with the results

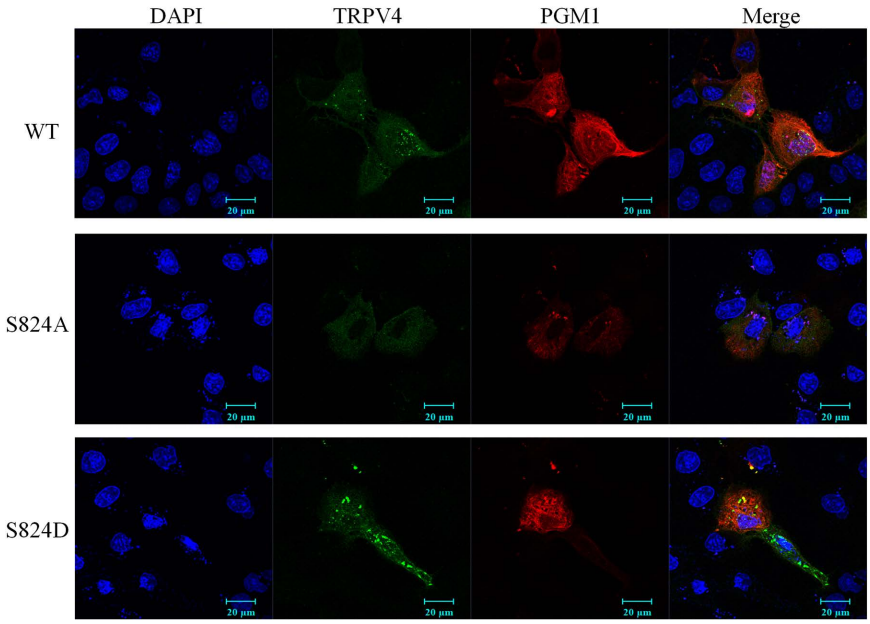


Figure 4. Confocal microscopic images of cells transfected with EGFP-TRPV4 WT, S824A, or S824D. Cells were examined by direct immunofluorescence microscopy (n = 5). Confocal microscopic images show EGFP-TRPV4 WT or mutant (S824A or S824D) (green), pmCherry-N1 PGM1 (red), and merged channels (yellow). EGFP-TRPV4 WT and S824D showed the greatest extent of colocalization with pmCherry-N1 PGM1 in the cytosol (upper and bottom panels). However, EGFP-TRPV4 S824A was primarily detected in the cytosol, and colocalized to a much lesser extent with PGM1(middle panel).

shown in **Figure 3**. Thus, these observations further support the hypothesis that phosphorylation on TRPV4 S824 regulates not only the interaction between TRPV4 and PGM1, but also subcellular localization of TRPV4.

3.5. Regulation of TRPV4 Subcellular Localization and Its Interaction with PGM1 by Phosphorylation of TRPV4 S824

We found previously that the C-terminal domain of TRPV4 interacts with either actin or tubulin, depending on the phosphorylation status of TRPV4 S824 [28]. As we demonstrated that PGM1 also interacts with the C-terminal domain of TRPV4, we hypothesized that a TRPV4 interaction with PGM1 is regulated by the phosphorylation status of the TRPV4serine residue 824. To determine whether TRPV4 localization and/or TRPV4 interaction with PGM1 are influenced by the phosphorylation status of TRPV4 S824, we examined localization of TRPV4 upon pharmacological treatment with GSK650394 (an SGK1 kinase inhibitor) or insulin (an SGK1 activator). As controls, TRPV4 S824A and S824D mutant versions were also used (**Figure 5(a)**). The subcellular locations of the TRPV4 mutants were not altered upon treatment with either GSK650394 or insulin, whereas the subcellular localization of the WT version was altered dramatically, according to the specific treatment (**Figure 5(a)**). The effect of PGM1 on TRPV4 activity was also determined by whole-cell Ca^{2+} image analysis in transiently-transfected HEK293 cells. As shown in **Figure 5(b)**, the S824D mutant version of TRPV4 enhanced Ca^{2+} current significantly, whereas the channel activity of TRPV4 WT or S824A was unaffected. This result suggests that PGM1 prefers to bind the activated (phosphorylated) TRPV4, resulting in plasma membrane localization.

These findings suggest that the phosphorylation status of TRPV4 S824 regulates both the binding of the C-terminal domain of TRPV4 with PGM1, and also subcellular localization of TRPV4 (plasma membrane or focal adhesions).

3.6. Putative Role of PGM1 in TRPV4 Ca^{2+} Regulation

We demonstrated the presence of PGM1 in the TRPV4 channel complex, indicating a possible function for

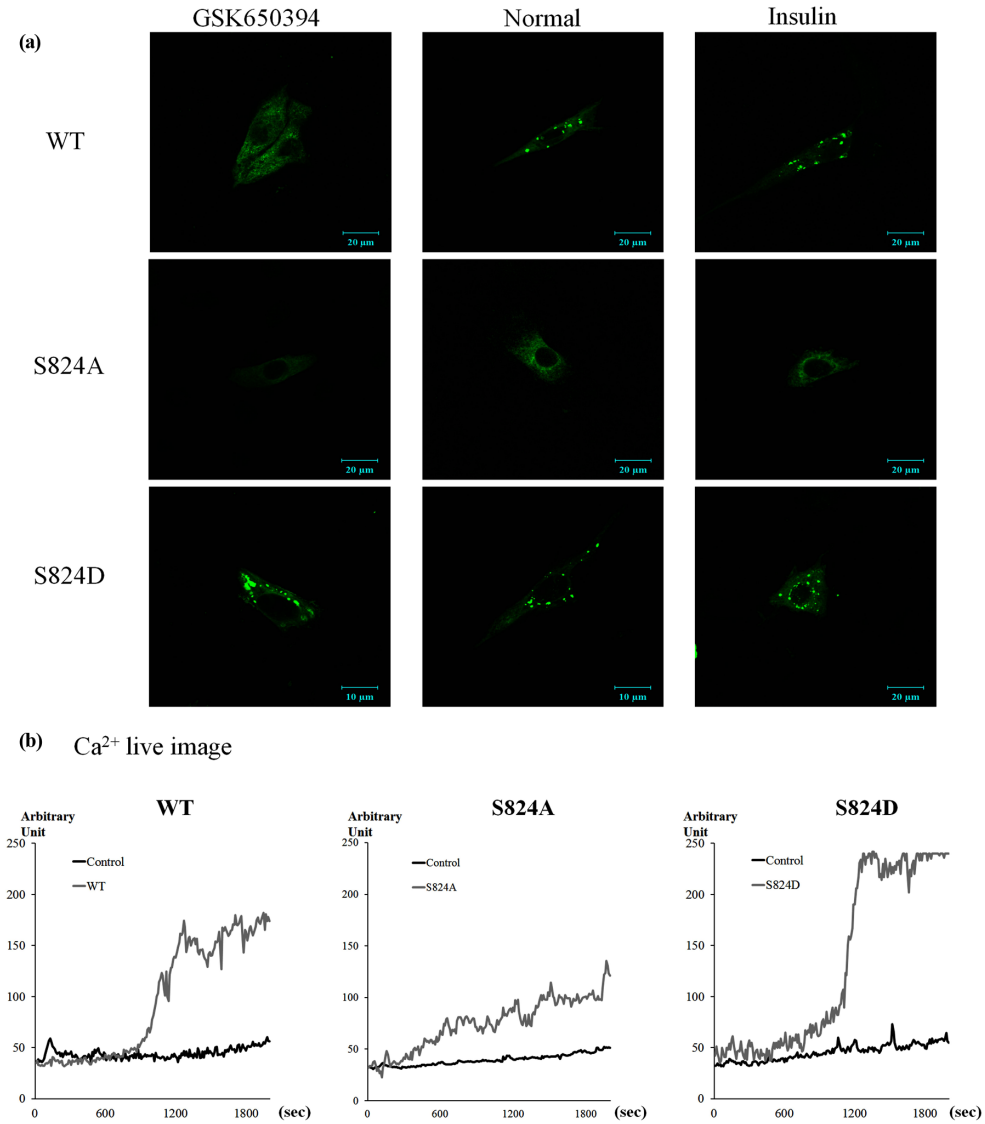


Figure 5. Plasma membrane localization of TRPV4 WT, S824A, or S824D in cells after treatment with GSK650394 or insulin. (a) Confocal microscopic analysis of cells transfected with EGFP-TRPV4 S824A, WT, or S824D (green) after treatment with an SGK1 inhibitor (GSK 650397, left), an SGK1 activator (insulin, right) for 12 h, or a serum-free control (middle). Subcellular localization of TRPV4 WT (which can be phosphorylated on S824 by SGK1) was altered by treatment with either GSK 650394 or insulin. The image shown is representative of five independent experiments (n = 5). (b) Effects of PGM1 on TRPV4 WT, S824A, or S824D, resulting from expression of TRPV4 WT, S824A, or S824D, expressed as absorption values at 488 nm from an argon-ion laser in HEK 293 cells (in arbitrary % units). After transiently co-transfecting the EGFP-TRPV4 WT or mutant (S824A or S824D) (green), pmCherry-N1 PGM1 (red), $[Ca^{2+}]_i$ was measured in single cells at 24˚C. Results shown are from one of five independent experiments.

PGM1 in the regulation of channel localization and/or activity. Because we demonstrated previously that S824 in TRPV4 is phosphorylated by SGK1, we propose a model in which the phosphorylation status of TRPV4 S824 enhances its interaction with PGM1, thereby resulting in inhibition of TRPV4 channel activity in the plasma membrane (**Figure 6**).

A bivalent metal ion, usually magnesium or cadmium (both of which complex directly with the phosphoryl group esterified to the PGM1 active site serine), is required for PGM1 enzymatic activity. Thus, it seems logical that association of PGM1 with the active Ca^{2+} channel (for example, via the S824-phosphorylated form of

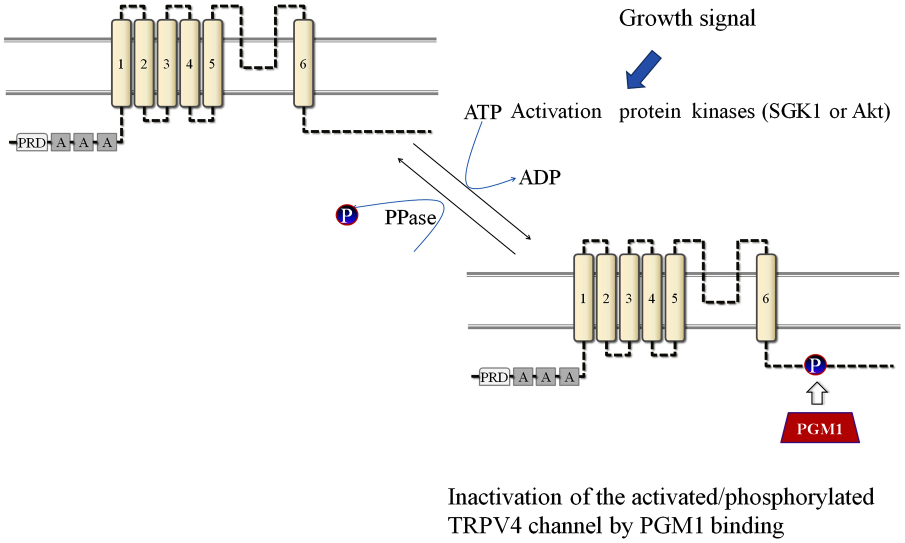


Figure 6. Putative model for the regulation of the interaction between TRPV4 and PGM1 by phosphorylation on TRPV4 S824. PGM1 can be activated by Ca^{2+}/Mg^{2+}, which is transported by activated TRPV4. PGM1 preferentially associates with the phosphorylated form of the TRPV4 C-terminal cytoplasmic domain; thus, blocking the Ca^{2+} channel. PGM1 may play a role in TRPV4 channel regulation, similar to that played by calmodulin (CaM), thereby generating a feedback inhibition loop.

TRPV4) would be necessary to enhance PGM1 activity. However, association of PGM1 with the activated Ca^{2+} channel actually appears to inhibit channel activation.

In conclusion, we provide the first evidence that PGM1 interacts with the C-terminal domain of TRPV4. We also present data to indicate a regulatory role for the PGM1-TRPV4 complex in Ca^{2+} reabsorption, in particular, via TRPV4 subcellular localization. The interaction between PGM1 and TRPV4 was regulated by the phosphorylation status of TRPV4 S824, resulting in enhanced channel activity. The molecular mechanism that we propose involves tethering of the PGM1-TRPV4 complex to the Ca^{2+} channel, thereby inactivating its channel activity. This mechanism is likely applicable to other ion transporters given the broad tissue distribution of TRPV4 and PGM1. Thus, phosphorylation of TRPV4 S824 appears to be a major regulatory mechanism for its interaction with many proteins, including PGM1, IP3R3, CaM, actin, tubulin, and STIM1 [29].

4. Discussion

We identified PGM1 as an auxiliary protein for the epithelial Ca^{2+} channel protein, TRPV4 (**Figure 1**). PGM1 catalyzes conversion of glucose 1-phosphate to glucose 6-phosphate, which is a useful metabolic intermediate [15]-[24]. Although the glucose 1-phosphate molecule is not a useful metabolic intermediate, PGM1 catalyzes conversion of glucose 1-phosphate to glucose 6-phosphate [23]. After glycogen phosphorylase catalyzes the phosphorolytic cleavage of a glucosyl residue from the glycogen polymer, the liberated glucose molecule retains a phosphate group on its 1-carbon atom [19]. The metabolic fate of glucose 6-phosphate depends on the needs of the cell at the time it is generated. If the cell is in a low-energy state, glucose 6-phosphate will be metabolized down the glycolytic pathway, eventually yielding two molecules of adenosine triphosphate [23]. If the cell is in need of biosynthetic intermediates, glucose 6-phosphate will enter the pentose phosphate pathway, where it will undergo a series of reactions that yield ribose and/or NADPH, depending on cellular conditions [29]. Thus, as PGM1 requires Ca^{2+}/Mg^{2+} for activation, it tends to localize near Ca^{2+} channels, such as TRPV4 or the vacuolar Ca^{2+}-ATPase, Pmc1p [28] [30]. In yeast, the role of Pmc1p appears to be similar to that of TRPV4 in human cells regarding PGM1 activation (**Figure 6**). Binding of PGM1 to the channel seems to block Ca^{2+} transport through the channel. However, it remains to be characterized whether the association with the activated or phosphorylated TRPV4 affects activity of PGM1 and the glycolytic pathway.

TRPV4 contains a similar aspartate-to-glutamate substitution at a different location in the selectivity filter, which may similarly account for somewhat lower P_{Ca}^{2+}/P_{Na}^{+}~6 selectivity compared with P_{Ca}^{2+}/P_{Na}^{+}~10 for

TRPV1 and TRPV3. Permeability values relative to Na^+ are 6 - 10 for Ca^{2+} and 2 - 3 for Mg^{2+} [31]. Their observations suggest that TRPV4 is a better channel for Mg^{2+} (which is required for PGM1 activation) than other TRP family members [32]. Furthermore, it was well characterized that TRPV4, which transports both Ca^{2+}/Mg^{2+} ions, is the only active channel within body temperature [1] [2] [7]. Based on these reasons, we assume that this channel is selected for the activator for PGM1. Therefore, the interaction between these two proteins (TRPV4 and PGM1) is a major clue to answer why the mutant TRPV4 causes many human genetic diseases, including metatropic dysplasia [1]-[4].

Our data provide the first evidence of a regulatory role for the PGM1-TRPV4 complex in Ca^{2+} reabsorption. Interaction between PGM1 and TRPV4 is also regulated by the phosphorylation status of TRPV4 S824 (**Figure 1(b)**). Thus, we suggest that phosphorylation of TRPV4 S824 is a major regulatory mechanism determining its interaction with many other proteins, including PGM1, STIM1, CaM, IP3R3, actin, and tubulin (**Figure 6**).

We demonstrated that the TRPV4 channel is an authentic substrate of SGK1, and that S824 of TRPV4 is phosphorylated by SGK1 (**Figure 1(b)**). Furthermore, we demonstrated that phosphorylation of S824 of TRPV4 is required for its interaction with F-actin, using TRPV4 mutants (S824D and S824A); this interaction also affected its subcellular localization. Here, we showed that phosphorylation of TRPV4 S824 promotes its association with PGM1; thus, inactivating TRPV4 (**Figure 6**). This signal convergence on the C-terminal domain of TRPV4 may constitute an important mechanism by which the timing and convergence of signal responses is integrated. Our results provide evidence for a functional role of plasma membrane-resident TRPV4 in the regulation of store-operated Ca^{2+} entry, mediated by protein-protein interactions of its C-terminal domain (**Figure 1(a)**).

We also reported that binding of Ca^{2+}-CaM is prevented by SGK1-mediated phosphorylation on S824, a residue located within the CaM binding site [27]. Conversely, substitution of the target residue (S824) with aspartic acid (S824D) results in a more rapid and sustained activation of TRPV4-mediated current. These data show that Ca^{2+}-CaM binding is inhibited by phosphorylation, indicating negative feedback regulation even at high Ca^{2+} concentrations. Even though phosphorylation of TRPV4 S824 was mediated by SGK1 (**Figure 1(b)**), this residue can also be phosphorylated by other protein kinases, including Akt kinase and protein kinase A. Further elucidation of the integration of extracellular signals with TRPV4 activation is required to better understand the mechanisms of TRPV4 regulation (**Figure 6**). TRPV4 plays an important role in pathological sensory perception and bone growth. As the potential effects of genetic mutations affecting TRPV4 function in human disease remain to be elucidated, characterization of TRPV4-interacting proteins may be useful for future efforts to cure or alleviate human diseases caused by TRPV4mutations.

CaM is a ubiquitously-expressed, dual-function protein that regulates the activity of multiple ion channels, Ca^{2+} pumps, and other proteins in a Ca^{2+}-dependent manner [2] [14]. Thus, we hypothesized that the association between PGM1 and TRPV4 inhibits TRPV4 channel activity in the plasma membrane. The activated nature of TRPV4 S824D seems to be achieved by preventing the binding of inhibitory proteins, such as Ca^{2+}-CaM or PGM1 (**Figure 4(b)**). As a bivalent metal ion, usually magnesium or cadmium (which both complex directly with the phosphoryl group esterified to the serine PGM1 active site) is required for PGM1 enzymatic activity, association of PGM1 with the active Ca^{2+} channel (via S824-phosphorylated TRPV4) is necessary for enhancing PGM1 activity (**Figure 6**). However, the association between PGM1 and the activated TRPV4 Ca^{2+} channel seems to inhibit channel function. TRPV4 contains a consensus sequence for SGK1-mediated phosphorylation within its CaM-binding domain (residues 811 - 850) (**Figure 1(b)**). The subcellular localization of TRPV4-S824A and S824D were independent of SGK1 activity (**Figure 5(a)**), whereas that of TRPV4-WT was affected. Thus, both PGM1 and CaM negatively affect TRPV4 channel function through protein-protein interactions (**Figure 6**). However, we do not know whether these two proteins affect TRPV4 function in a synergistic or competitive manner. As additional and as-yet unidentified proteins may also modulate TRPV4 function through protein-protein interactions with its C-terminal domain, further characterization of proteins interacting with this domain will be helpful to better understand its role (**Figure 1**). It also remains to be clarified whether the C-terminal domains of other TRP channel proteins (in addition to TRPV4) interact with PGM1. Because TRPV4 channels are often composed of heteromeric subunits *in vivo*, PGM1 may form a protein complex with other TRP channels.

5. Conclusion

The C-terminal domain of transient receptor potential vanilloid 4 (TRPV4) interacts with human phosphoglu-

comutase-1(PGM1). Phosphorylation on TRPV4 S824 enhances its association with PGM1. TRPV4-PGM1 interaction which is enhanced by the phosphorylation on TRPV4 serine 824 residue modulates its channel activity.

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Haematological Traits of Nigerian Indigenous Pig and Its Hybrid (50% Large White × 50 NIP) at Post Weaning Ages

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Abstract

Pigs play a major role in socioeconomic life of the people; it serves as a source of income for rural population and fulfill important role in culture and food security. Fifty-one weaners were randomly selected at 9, 17 and 25 weeks of age for analysis. Blood from the jugular vein of the weaners was taken to the laboratory for analysis. The leaukocyte parameters used for this study were White blood cell count (WBC), Lymphocyte (LYM) count, Neutrophil (NEU) count, Eosinophil (EOS) and Monocyte (MON) count) and Platelet (PLT) count. Data collected on these leaukocyte parameters were subjected to Analysis of Variance (ANOVA) while the means were separated using Duncan's Multiple Range Test. The results showed that at 9 weeks of age, compared with the female hybrid, the male hybrid had higher PCV, NEU and EOS (33.12% ± 2.73%, 31.00% ± 3.37% and 0.38% ± 0.18% respectively) values, while the female NIP had higher WBC, LYM and MON (24.55 ± 1.09 × 10^3/µl, 56.00% ± 3.24% and 0.50% ± 0.50%) values respectively. At 17 weeks of age, the male hybrid had higher WBC and NEU (32.18 ± 1.92 × 10^3/µl and 32.38% ± 3.56%) values while the female had higher PCV, LYM, EOS and MON (42.50% ± 1.50%, 63.00% ± 5.0%, 2.50% ± 2.50% and 4.50% ± 1.50%) values. At 25 weeks of age, the female hybrid had higher values in all the parameters with the exception of NEU and MON where male had higher values. These ages can be used as criteria for selection for immunocompetence in pigs.

Keywords

Nigerian Indigenous Pigs, Hybrid, Leukocyte Traits, Post Weaning Age

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1. Introduction

The pig population in Nigeria is about 7 million in 1997, where most of which are raised on smallholder farms [1]. They are concentrated mainly in the Middle and Southern States of the country due to religious, cultural and local taboo. One of the important indicators of health and disease in animals is the haematological profile. It is very important in diagnosis, treatment or prognosis of many diseases [2]. Determination of the haematological profiles reflects the physiological responsiveness of the animals to its internal and external environments [3]. Radostits *et al.* (1994) [4] reported that the intensification of animal agriculture had created complex animal health and production of more problems for which there were no simple and reliable therapeutic and preventive procedures. These conditions adversely affect the health or welfare of the animals and impair their homeostatic mechanisms resulting in the body dysfunction which may be fatal [5].

The blood consisting of blood cells and plasma are responsible for transportation, regulation, protection and homeostatic functions in the animal body [6]. Anaemia has been described for many diseases and management problems and physiological state such as age in pigs. Determination of the Packed Cell Volume (PCV), erythrocyte count (RBC) and haemoglobin (Hb) can give indications of the level of anaemia in the animal. Haematological parameters of pig have been widely studied and have been reported to vary depending on sex, age, geographical location and experimental procedures [4] [7]-[9]. This study is to provide the empirical evidence needed for the conservation and utilization of NIP and its crossbred in swine improvement programme. The specific objective is to evaluate growth and leukocyte traits of NIP and its hybrid at post-weaning ages of 9, 17 and 25 weeks.

2. Materials and Methods

Fifty-one weaners, from breeding plan in **Table 1**, were randomly selected at 9, 17 and 25 weeks of age for

Table 1. Effect of genotype and age on leucocyte traits.

Age	Traits	Breed	
		Hybrid	NIP
9 weeks	***PCV (%)**	**32.30 ± 2.45^{b}**	**40.62 ± 0.65^{a}**
	***WBC (×10^{3}/µl)**	**25.64 ± 2.63^{a}**	**22.78 ± 1.09^{b}**
	***NEU (%)**	**27.30 ± 3.63^{b}**	**41.38 ± 3.01^{a}**
	***LYM (%)**	**72.30 ± 3.65^{a}**	**57.12 ± 2.87^{b}**
EOS (%)		0.30 ± 0.15	1.00 ± 0.33
MON (%)		0.10 ± 0.10	0.50 ± 0.33
17 weeks	PCV (%)	41.90 ± 0.43	41.62 ± 1.34
	WBC (×10^{3}/µl)	32.96 ± 1.76	32.96 ± 1.72
	***NEU (%)**	**31.90 ± 2.96^{b}**	**41.88 ± 3.68^{a}**
	***LYM (%)**	**63.10 ± 2.95^{a}**	**53.12 ± 3.97^{b}**
EOS (%)		1.90 ± 0.90	2.75 ± 0.90
MON (%)		3.10 ± 0.6	2.25 ± 0.96
25 weeks	***PCV (%)**	**38.86 ± 1.26^{b}**	**43.88 ± 1.26^{a}**
	WBC (×10^{3}/µl)	35.54 ± 1.87	37.70 ± 1.87
NEU (%)		32.43 ± 2.84	32.88 ± 2.61
LYM (%)		61.00 ± 2.59	60.38 ± 2.86
EOS (%)		2.00 ± 0.5	83.00 ± 0.95
MON (%)		4.86 ± 0.4	64.25 ± 0.50

^{a,b}Mean values for each trait along row with different superscript letters, are different. *Level of significance ($P < 0.05$). PCV—Packed cell volume, WBC—White blood cell, NEU—Neutrophil, LYM—Lymphocyte, EOS—Eosinophil, MON—Monocytes, NIP—Nigerian Indigenous Pig.

haematological analysis. Blood from the jugular vein of these pigs were collected into anti-coagulant bottles and taken to the laboratory for analysis. The blood parameters taken were packed cell volume (PCV%) and leukocyte traits (white blood cell count (WBC), neutrophilic count (NEU%), eosinophil percentage (EOS%), lymphocyte percentage (LYM%), and monocytes percentage (MON%)). The PCV was determined using the microhaematocrit method while leucocyte counts were determined using the improved Neubauer counting chamber [8].

2.1. Packed Cell Volume (PCV)

75 × 16 mm capillary tubes were filled with blood and centrifuged at 3000 rpm for 5 minutes. The haematocrit-values were determined with the aid of a microhaematocrit reader.

2.2. White Blood Cell Counts

Blood was well mixed on a roller mixer and 1:20 dilution of blood was made by adding 20 µl (0.02 ml) of pigs blood to 0.38 ml of diluting fluid (2% glacial acetic acid in distilled water (Turk's solution) coloured pale violet with 1% gentian violet) in a 75 × 10 mm tube. The suspension was well mixed and allowed to stand for 1 minute. The cells were counted in the four corner square millimeters of the charged chamber (Improved Neubauer counting chamber) under 10× magnification of a microscope.

2.3. Statistical Analysis

Haematological data recorded were analyzed by using Analysis of variance (ANOVA) and means were separated by Duncan Multiple Range Test (DMRT).

3. Results

The means of leukocyte traits of hybrid at 9, 17 and 25 weeks respectively are as follows; PCV (32.30% ± 2.45%, 41.90% ± 0.43%, 38.86% ± 1.26%); WBC (25.64 ± 2.63 ($\times 10^3$/µl), 32.96 ± 1.76 ($\times 10^3$/µl), 35.54 ± 1.87 ($\times 10^3$/µl)); NEU (27.30% ± 3.63%, 31.90% ± 2.96%, 32.43% ± 2.84%); LYM (72.30% ± 3.65%, 63.10% ± 2.95%, 61.00% ± 2.59%); EOS (0.30% ± 0.15%, 1.90% ± 0.90%, 2.00% ± 0.58%) and MONO (0.10% ± 0.10 %, 3.10% ± 0.6%, 4.86% ± 0.46%). The mean for NIP at 9, 17 and 25 weeks respectively are as follows; PCV (40.62% ± 0.65%, 41.62% ± 1.34%, 43.88% ± 1.26%); WBC (22.78 ± 1.09 ($\times 10^3$/µl), 32.96 ± 1.72 ($\times 10^3$/µl), 37.70 ± 1.87 ($\times 10^3$/µl)); NEU (41.38% ± 3.01%, 41.88% ± 3.68%, 32.88% ± 2.61%[a]); LYM (57.12% ± 2.87%, 53.12% ± 3.97%, 60.38% ± 2.86%); EOS (1.00% ± 0.33%, 2.75% ± 0.90%, 3.00% ± 0.95%) and MONO (0.50% ± 0.33%, 2.25% ± 0.96%, 4.25% ± 0.50%).

The PCV was significantly different ($P < 0.05$) between hybrid and NIP at 9 and 25 weeks of age while there was no significant difference ($P > 0.05$) at 17 weeks of age. The Leukocyte count, NEU and LYM, were significantly different ($P < 0.05$) between hybrid and NIP at 9 and 17 weeks of age while there was no significant difference ($P > 0.05$) at 25 weeks of age. It was observed that the LYM values for hybrid were higher than that of NIP while the NEU value for NIP were higher than that of hybrid throughout the ages considered (9, 17 and 25 weeks). This is presented graphically in **Figure 1**.

The effect of sex and age on leukocyte traits for the hybrid and NIP at 9, 17 and 25 weeks of age are presented in **Table 2** and **Table 3** respectively.

The PCV of female and male hybrid were significantly different ($P < 0.05$) from each other at 9 weeks (29.00% ± 4.00% and 33.12% ± 2.73%) and 25 weeks (42.00% ± 0.00% and 38.33% ± 1.38%) of age. Also at 9 and 25 weeks of age, the Leukocyte traits, WBC (28.90 ± 4.60 ($\times 10^3$/µl), 24.83 ± 3.15 ($\times 10^3$/µl and 41.00 ± 0.00 ($\times 10^3$/µl) and 34.63 ± 1.94 ($\times 10^3$/µl)), NEU (12.50% ± 1.50% and 31.00% ± 3.37%, 22.00% ± 0.00% and 34.17% ± 2.66%) and LYM (87.00% ± 1.00% and 68.63% ± 3.43%, 72.00% ± 0.00% and 59.17% ± 2.17%), were significantly different ($P < 0.05$). The female and male hybrid MONO (3.00% ± 0.00% and 5.17% ± 0.40%) and EOS (3.00% ± 0.00% and 4.83% ± 0.65%) were significantly different ($P < 0.05$) at 25 weeks of age.

The NEU and LYM of NIP females and males were significantly different throughout the ages except at 17 weeks of age where the LYM was not significantly different ($P > 0.05$). The WBC was also significantly different at 9 and 17 weeks while it was not significant at 25 weeks of age. This effect is presented graphically in **Figures 2-4**. The PCV and NEU values were higher in NIP males and females at 9 weeks but were comparable

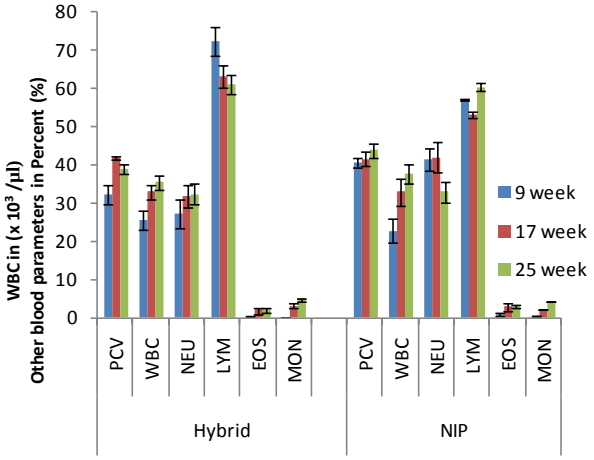

Figure 1. Effect of genotype on the haematological traits at 9, 17 and 25 weeks. PCV—Packed cell volume, WBC—White blood cell, NEU—Neutrophil, LYM—Lymphocyte, EOS—Eosinophil, MON—Monocytes, NIP—Nigerian Indigenous Pig.

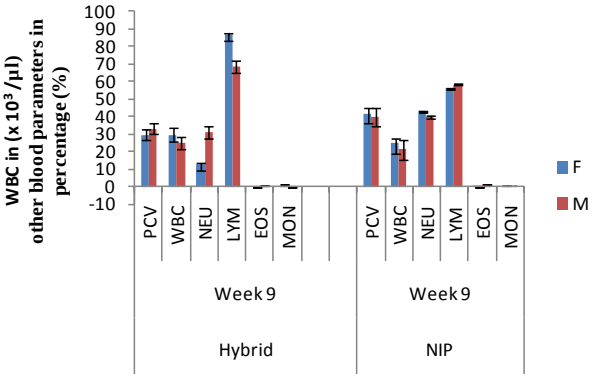

Figure 2. Effect of genotype and sex on white blood cell count at 9 weeks. PCV—Packed cell volume, WBC—White blood cell, NEU—Neutrophil, LYM— Lymphocyte, EOS—Eosinophil, MON—Monocytes, M—Male, F—Female, NIP—Nigerian Indigenous pig.

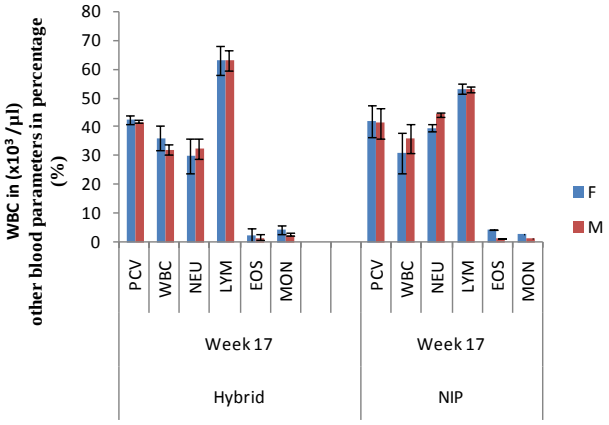

Figure 3. Effect of genotype and sex on white blood cell count at 17 weeks. PCV—Packed cell volume, WBC—White blood cell, NEU—Neutrophil, LYM—Lymphocyte, EOS—Eosinophil, MON—Monocytes, M—Male, F—Female, NIP—Nigerian Indigenous Pigs.

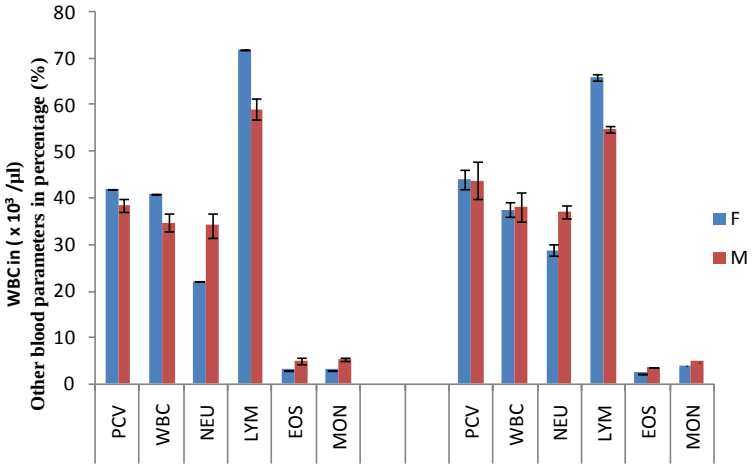

Figure 4. Effect of genotype and sex on white blood cell count at 25 weeks. PCV—Packed cell volume, WBC—White blood cell, NEU—Neutrophil, LYM—Lymphocyte, EOS—Eosinophil, MON—Monocytes, M—Male, F—Female, NIP—Nigerian Indigenous pig.

Table 2. Table type styles (Table caption is indispensable).

Age	Traits	Female	Male
9 weeks	*PCV (%)	**29.00 ± 4.00^{b}**	**33.12 ± 2.73^{a}**
	*WBC	**28.90 ± 4.60^{a}**	**24.83 ± 3.15^{b}**
	*NEU (%)	**12.50 ± 1.50^{a}**	**31.00 ± 3.37^{b}**
	*LYM (%)	**87.00 ± 1.00^{a}**	**68.63 ± 3.43^{b}**
	EOS (%)	0.00 ± 0.00	0.38 ± 0.18
	MON (%)	0.50 ± 0.50	0.00 ± 0.00
17 weeks			
	PCV (%)	42.50 ± 1.50	41.75 ± 0.45
	WBC (×10^{3}/µl)	36.10 ± 4.20	32.18 ± 1.92
	NEU (%)	30.00 ± 6.00	32.38 ± 3.56
	LYM (%)	63.00 ± 5.00	63.12 ± 3.61
	EOS (%)	2.50 ± 2.50	1.75 ± 1.03
	MON (%)	4.50 ± 1.50	2.75 ± 0.65
25 weeks			
	*PCV (%)	**42.00 ± 0.00^{a}**	**38.33 ± 1.38^{b}**
	*WBC (×10^{3}/µl)	**41.00 ± 0.00^{a}**	**34.63 ± 1.94^{b}**
	*NEU (%)	**22.00 ± 0.00^{b}**	**34.17 ± 2.66^{a}**
	*LYM (%)	**72.00 ± 0.00^{a}**	**59.17 ± 2.17^{b}**
	*EOS (%)	**3.00 ± 0.00^{b}**	**4.83 ± 0.65^{a}**
	*MON (%)	**3.00 ± 0.00^{b}**	**5.17 ± 0.40^{a}**

^{a,b}Mean within the same row with different superscript letters, were different ($P < 0.05$). *Level of significance ($P < 0.05$). PCV—Packed cell volume, WBC—White blood cell, NEU—Neutrophil, LYM—Lymphocyte, EOS—Eosinophil, MON—Monocytes, NIP—Nigerian Indigenous Pig.

Table 3. Effect of sex and age on Leucocyte traits of Hybrid.

	Age	Traits	Female	Male
	9 weeks	***PCV (%)**	**41.50 ± 0.65^{a}**	**39.75 ± 1.03^{b}**
		***WBC (×10^{3}/µl)**	**24.55 ± 1.09^{a}**	**21.00 ± 1.50^{b}**
		***NEU (%)**	**43.00 ± 3.03^{a}**	**39.76 ± 5.59^{b}**
		***LYM (%)**	**56.00 ± 3.24^{b}**	**58.25 ± 5.30^{a}**
		***EOS (%)**	**0.05 ± 0.50^{b}**	**1.50 ± 0.79^{a}**
MON **(%)**	0.50 ± 0.50	0.50 ± 0.50		
	17 weeks			
		PCV **(%)**	42.00 ± 1.08	41.25 ± 2.66
		***WBC** (×10^{3}/µl)	**30.88 ± 1.81^{b}**	**35.90 ± 2.54^{a}**
		***NEU (%)**	**39.50 ± 5.62^{b}**	**44.25 ± 5.27^{a}**
LYM **(%)**	53.25 ± 6.97	53.00 ± 5.00		
		***EOS (%)**	**4.25 ± 1.32^{a}**	**1.25 ± 0.75^{b}**
MON **(%)**	3.00 ± 1.73	1.50 ± 0.96		
	25 weeks			
PCV **(%)**	44.00 ± 0.41	43.75 ± 1.03		
		WBC (×10^{3}/µl)	37.43 ± 2.96	37.98 ± 1.10
		***NEU (%)**	**28.75 ± 2.14^{b}**	**37.00 ± 3.98^{a}**
		***LYM (%)**	**66.00 ± 1.58^{a}**	**54.75 ± 3.18^{b}**
EOS **(%)**	2.30 ± 1.32	3.50 ± 1.50		
MON **(%)**	3.75 ± 0.75	4.75 ± 0.75		

^{a,b}Mean within the same row with different superscript letters, were different ($P < 0.05$). *significance ($P < 0.05$). PCV—Packed cell volume, WBC—White blood cell, NEU—Neutrophil, LYM—Lymphocyte, EOS—Eosinophil, MON—Monocytes.

at 17 and 25 weeks of age with those of the hybrid. The LYM values were higher for Hybrid males and females over the NIP males and females for all ages.

Table 4 and **Table 5** show Pearson's correlation coefficient among the leukocyte traits of hybrids and NIPs respectively. There was correlation among the white blood count, the hybrid correlation values were within −0.04 to 0.64 while NIP correlation values were within −0.02 to 0.36. The highest correlation was found between the PCV and WBC with 0.64 values in hybrid as shown in **Table 4** while it was 0.36 between PCV and WBC for NIP as shown in **Table 5**. In **Table 4** and **Table 5**, the correlation values for hybrid and NIP for WBC and Monocytes (0.58, 0.46) and NEU and LYM (−0.95, −0.93) were highly significant ($P < 0.05$). This positive correlation between WBC and MONO, insinuated that the increase in WBC will cause an increase in MONO while the negative correlation between Neutrophil and Lymphocyte mean that an increase in LYM will cause a decrease in Neutrophil.

4. Discussion

The significant variations due to age and breed were observed in this study. This finding was corroborated with Sutherland *et al.* (2005) [10] and Aladi *et al.* (2008) [9] where the interaction between pig breed and age were significant during both pre and post weaning age of different breeds of pigs. This variation may be due to genetic control of the individual pigs as described by Renard *et al.*, (1998). The significant effects of age and sex on haematolological parameters, especially, PCV, NEU and LYM, were in collaboration with findings of Radositis *et al.*, 1994 [4] and Egbunike and Ekusu (1983) [7], where sex and age influence haematological values in pigs. According to Egbunike and Ekusu (1983) [7] who reported that the male tend to have higher values than the female. Okeudo *et al.* (2003) [11] also reported sex-related variation in ducks and guinea fowls. This finding was in contradiction with findings of Aladi *et al.* (2008) [9] and Eze *et al.* (2010) [12].

Table 4. Pearson correlation coefficients among the white blood count of hybrid.

	PCV	WBC	NEU	LYM	EOS	MON
PCV	1.00	0.64	0.52	−0.60	0.18	0.41
WBC		1.00	0.15	−0.33	0.31	*__0.58__
NEU			1.00	*__−0.95__	−0.04	0.20
LYM				1.00	−0.21	−0.25
EOS					1.00	0.29
MON						1.00

*Level of significance P < 0.05. PCV—Packed cell volume, WBC—White blood cell, NEU—Neutrophil, LYM—Lymphocyte, EOS—Eosinophil, MON—Monocytes.

Table 5. Pearson correlation coefficient among the white blood count of NIP (P < 0.05).

	PCV	WBC	NEU	LYM	EOS	MON
PCV	1.00	*__0.36__	*__0.02__	*__−0.09__	*__0.02__	0.31
WBC		1.00	−0.18	0.03	0.25	*__0.46__
NEU			1.00	*__−0.93__	−0.14	−0.24
LYM				1.00	−0.15	*__−0.06__
EOS					1.00	0.20
MON						1.00

*Level of significance P < 0.05. PCV—Packed cell volume, WBC—White blood cell, NEU—Neutrophil, LYM—Lymphocyte, EOS—Eosinophil, MON—Monocytes.

The NIP had higher value of PCV at 9 and 25 weeks (40.62% ± 0.65% and 43.88% ± 1.26%) respectively than Hybrid value (32.30% ± 2.45% and 38.86% ± 1.26%). This finding is corroborated with Aladi *et al.* (2008). The NEU and LYM increasing at 17 weeks and then decreasing at 25 weeks of age, was in line with Aladi *et al.* (2008) [9]. The NIP PCV increased with age. This is contrary to Aladi *et al.* (2008) [9] findings where the NIP PCV decreases with age.

It was observed from this study that the NIP PCV and the WBC were significantly correlated (P < 0.05) where both increased with age while it was not for hybrid. The negative correlation between NEU and LYM that was significant (P < 0.05) in both breed was observed to be true in hybrid where the NEU increased, LYM decreased with age while in NIP as the NEU increased from 9 weeks to 17 weeks, it then decreased at 25 weeks; while the LYM decreased from 9 weeks to 17 weeks and then increased at 25 weeks.

The WBC that is higher than the normal value for all pigs ($7 - 20 \times 10^3$) must be as a result of its contact with pathogen in which the immune system produces more white blood cells to combat the foreign body. This result is in agreement with Eze *et al.*, (2010) [12] where the WBC of the pigs increases above the standard WBC for pigs. White blood cells are important humoral immune cells in blood circulation and they can recognize antigen, kill antigen, clear and circulate immune complexes, they are also involved in immune regulation and have a complete self-regulation system. It is one of the first signs or indicators of disease invasion in animal body. Lunney and Rowland (2011) [13] reported that blood immune traits against diseases could be detected early. This early detection of against diseases through the blood immune traits can be used as criteria for selection at these ages. Leukocyte traits are one of the aspects of innate immune traits which are helpful step towards investigation into any link to disease resistance capacity (immunocompetence) and improve a measure to advance general health in the pig. In order to include immunocompetence in selection for improved health, a major challenge will be to correlate variation in heritable immune traits in healthy animals with inter-individual variability in response to various pathogens. Testing this hypothesis will be a key point for further use of immune traits as indirect selection criteria in multitrait selection to improve resistance to disease.

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Comparison of African Swine Fever Virus Prevalence in Nigerian Indigenous Pig, Its Hybrid and Backcross in an Environment Prone to African Swine Fever

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Abstract

This work investigated on the presence of African swine fever virus (ASFV) in Nigerian Indigenous pig (NIP), its hybrid and backcross using Polymerase chain reaction (PCR) screening method on the extracted DNA and haematological screening from these pig bloods. Pig populations selected from Southwestern Nigeria were used for this study. ASFV infected blood samples collected from the University of Ibadan were used as positive control. White blood cell count was significantly highest in hybrid (31.27 ± 1.79 × 10^3/µl) and higher in backcross (27.71 ± 2.01 × 10^3/µl) compared with NIP (18.16 ± 3.01 × 10^3/µl) for NIP, and while Lymphocyte count was found to be highest in hybrid (86.17% ± 1.95%) and lowest in backcross (56.23% ± 1.17%). The PAS primers (PAS_1F: 5'-ATG GAT ACC GAG GGA ATA GC-3' and PAS_2R: 5'-CTT ACC GAT GAA AAT GAT AC-3') amplified the 278 bp of ASFV in the DNA extracted from NIP, its hybrids and backcross. In conclusion, this study has shown that NIP, its hybrid and backcross have ASFV in their genome in an ASF-prone environment and thus confirming the continuous prevalence of ASF in Southwest Nigeria. This is an on-going research where the severity and virulence of the virus has to be measured.

Keywords

Pig, Polymerase Chain Reaction, African Swine Fever

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1. Introduction

Pigs play a major role in socioeconomic life of the people; it serves as a source of income for rural population and fulfill important role in culture and food security. The population of pig in Nigeria increases from 2 million in 1984 to 7 million in 1997 before the widespread of ASF epizootic [1].

Disease is one of the factors that affect the livestock production in Nigeria [2]. Jovanoic *et al*., 2009 [3] stated that diseases can have a significant impact on animal productivity and production, human health and, consequently, on the overall process of economic development Pigs harbor a range of parasites and diseases some of which are zoonotic. One of these is the African Swine Fever that is caused by a virus.

African swine fever (ASF) is a highly contagious viral disease of pigs and of such concern that it is included among the List A diseases by the United Nations Office International des Epizooties (OIE) [4]-[8]. It causes a devastating haemorrhagic fever of pigs with mortality rates approaching 100% with the acute and peracute forms. It causes major economic losses, threatens food security and limits pig production in affected countries. The disease causes significant economic losses in affected countries due to the high mortality rates associated. The transmission of the disease, as it now occurs in sub-Saharan Africa, is through the African soft tick (Ornithodoros moubata porcinus) and Warthogs or domestic pigs. The transmission through the warthog and soft ticks does not occur in West Africa, although ASF virus has been detected from Warthog in Nigeria of West Africa [9] and also in local pigs in Nigeria [10]. The presence of soft ticks is also confirmed in Warthogs [7].

The diagnosis of infectious diseases such as ASF can be performed by direct and/or indirect detection of infectious agents. By direct methods, the particles of the agents and/or their components, such as nucleic acids, structural or non-structural proteins, enzymes, etc., are detected. The indirect methods demonstrate the antibodies induced by the infections [11]. The most common direct detection methods are isolation or *in-vitro* cultivation, electron microscopy, immunofluorescence, immunohistochemistry, antigen enzyme-linked immunosorbent assay (antigen-ELISA), nucleic-acid hybridisation (NAH), macro- and microarrays and the various techniques of nucleic acid amplification, such as the polymerase chain reaction (PCR) or the isothermal amplification methods, such as Nucleic Acid Sequence Based Amplification (NASBA), Invader or Loop-Mediated Isothermal Amplification (LAMP). This study aimed at comparing the ASFV prevalence in NIP, its hybrid and backcross in an environment prone to African Swine Fever.

2. Materials and Method

2.1. Blood Sampling

Blood samples were collected from the jugular vein of pure NIP (20), crossbreds (10) and Backcross [14] into 5 ml sterile EDTA bottles and brought into the laboratory for haematological analyses and DNA extraction for Polymerase Chain Reaction (PCR).

2.2. Blood Analysis

Hematological traits which consist of mainly three components, including leukocyte traits, erythrocyte traits and platelet traits were measured. Blood parameters include leukocyte traits such as White Blood Cell Count (WBC), neutrophil (NEU), lymphocyte count (LY), monocytes (MONO), erythrocyte traits (Red Blood Cell (RBC), Hemoglobin (HGB), Hematocrit or Packed cell volume (PCV), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), Mean Corpuscular Hemoglobin Concentration (MCHC) and platelet trait (blood Platelet counts (PLT). All these blood routine parameters were measured as described by Makinde *et al.* (1991) [12], Mafuvadze and Erlwanger (2007). The MCV, MCHC and MCH were calculated from PCV, RBC and Hb data as follows; MCV = PCV/RBC, MCHC = Hb/PCV, MCH = Hb/RBC.

2.3. DNA Extraction

The DNA extraction was done by using DNA kit (Zymobeads). The manufacturer instructions were followed in details.

2.4. PCR Condition

Diagnostic Polymerase Chain Reaction was performed according to the Manual of Diagnostic Tests and Vacc-

ines (OIE, 2008). ASF-specific primers (oligonucleotide primers) targeting the major capsid protein (VP72 gene) amplifying a 278-bp fragment within the conserved region was employed: PAS_1F: 5'-ATG GAT ACC GAG GGA ATA GC-3' and PAS_2R: 5'-CTT ACC GAT GAA AAT GAT AC-3' (Luther *et al*., 2007; OIE, 2008). The final reaction volume of 25 µl PCR master mix comprised 10 µl extracted DNA template, 10 µl Nuclease Free Water, 10 µl oligonucleotide primers (for both Forward and Reverse) and 12 µl of already prepared Master Mix. Each tube was placed in an automated PCR thermal cycler (MG48+; Mygene™ Series) for amplification for 35 cycles as follows: initial denaturation at 94˚C for 3 minutes for 35 cycles, with 3 steps of denaturation at 94˚C for 30 seconds, annealing at 57˚C for 45 seconds and extension at 72˚C for 30 seconds and final extension at 72˚C for 5 minutes. Amplification products were analyzed by electrophoresis on a 1% agarose gel containing 0.5 µg of ethidium bromide per ml. The gel were visualized under Ultra violet light and photographed.

3. Results and Discussion

3.1. ASFV Detection

PAS Primers of ASFV amplify at 278 bp for infected pigs used as positive control, NIP, hybrid and the crossbred. **Plate 1** shows single and discrete band of ASFV amplification at 278 bp for NIP and for a positive control sample obtained from the Veterinary Medicine of the University of Ibadan while no band was observed for the negative control.

3.2. Haematological Status of the NIPs, Hybrid and Backcrosses

Haematological traits include Leukocyte related traits, red blood cells related traits and platelets traits, which are important components of the animal's immune system. The effect of genotype on the haematological parameters of NIP, Hybrid and Backcross is shown in **Table 1**. The means of Red Blood Count traits of NIP, hybrid and BC are respectively as follows: PCV (37.07% ± 1.50%, 33.39% ± 1.49%, 31.31% ± 0.87%); Hb (12.37 ± 0.50 (g/dl), 11.11 ± 0.50 (g/dl), 10.42 ± 0.29 (g/dl)); RBC (4.41 ± 2.02 ($\times 10^6$/ul), 5.31 ± 3.48 ($\times 10^6$/ul), 4.65 ± 3.56 ($\times 10^6$/ul)); MCV (87.45 ± 0.00 fl, 68.23 ± 0.01 fl, 74.52 ± 0.01 fl); MCHC (33.00 ± 0.00 (g/dl), 33.25 ± 0.00 (g/dl), 33.27 ± 0.00 (g/dl)) and MCH (29.15 ± 0.00 (g/dl), 22.70 ± 0.00 (g/dl), 24.80 ± 0.00 (g/dl)). The means of Leaukocyte traits of NIP, hybrid and BC are respectively as follows: WBC (18.16 ± 3.01 ($\times 10^3$/µl), 31.27 ± 1.79 ($\times 10^3$/µl), 27.71 ± 2.01 ($\times 10^3$/µl)); NEU (18.75 ± 1.77, 12.04 ± 1.87, 40.39 ± 1.17); LYM (77.89 ± 1.79, 86.17 ± 1.95, 56.23 ± 1.17); MONO (1.64% ± 0.21%, 1.04% ± 0.95%, 1.54% ± 0.29%) and EOS (2.21% ± 0.35%, 0.70% ± 0.25%, 1.92% ± 0.27%). The means of Platelet trait of NIP, hybrid and BC are respectively as follows: PLT (32.00 ± 2.08 ($\times 10^4$/ul), 59.56 ± 7.44 ($\times 10^4$/ul), 16.41 ± 2.20 ($\times 10^4$/ul)). It was observed that effect of breed was significant ($P < 0.05$) in all the Leukocyte-related traits with the exception of the Monocyte. Hybrid had higher values than NIP and Backcross in WBC and LYM with 31.27% and 86.17% values respectively. The backcrosses had higher value of 40.39% ± 1.17% in Neutrophil compared to hybrid and NIP while the NIP had higher values of Monocytes and Eosinophil (1.64% ± 0.21% and 2.21% ± 0.35%) compared to hybrid

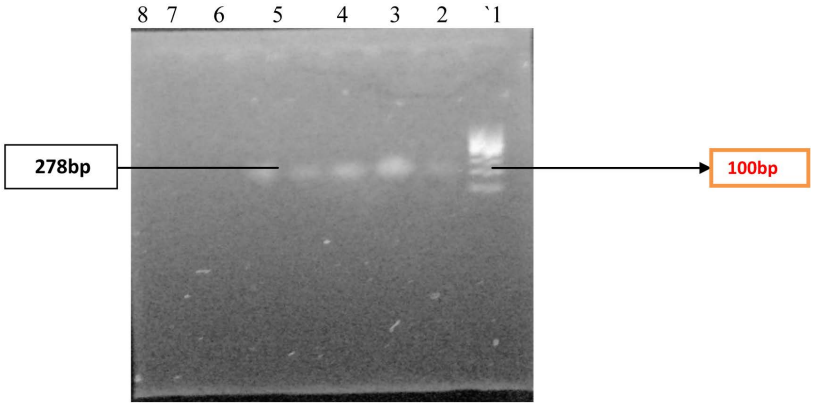

Plate 1. ASFV amplification in NIP DNA using PAS primer (278 bp) in lanes 2 - 5; lane 6 is the positive control; lane 7 is negative control. Molecular marker (100 bp) in Lane 1.

Table 1. Effect of genotype on the haematological parameters of NIP, Hybrid and Backcross.

Variables		Mean		
		NIP	Hybrid	Backcross
n		28	23	13
Red blood cell count	*PCV (%)	37.07 ± 1.50a	33.39 ± 1.49ab	31.31 ± 0.87b
	*Hb ((g/dl))	12.37 ± 0.50a	11.11 ± 0.50ab	10.42 ± 0.29b
	RBC (10^6/ul)	4.41 ± 2.02	5.31 ± 3.48	4.65 ± 3.56
	*MCV ((fl))	87.45 ± 0.00a	68.23 ± 0.01ab	74.52 ± 0.01b
	MCHC (g/dl)	33.00 ± 0.00	33.25 ± 0.00	33.27 ± 0.00
	*MCH (pg)	29.15 ± 0.00a	22.70 ± 0.00b	24.80 ± 0.00ab
White blood count	*WBC ($\times10^3$/µl)	18.16 ± 3.01b	31.27 ± 1.79a	27.71 ± 2.01c
	*NEU (%)	18.75 ± 1.77b	12.04 ± 1.87c	40.39 ± 1.17a
	*LYM (%)	77.89 ± 1.79b	86.17 ± 1.95a	56.23 ± 1.17c
	MON (%)	1.64 ± 0.21	1.04 ± 0.95	1.54 ± 0.29
	*EOSI	(%)	2.21 ± 0.35a	0.70 ± 0.25b
Platelet	*PLT ($\times10^4$/ul)	32.00 ± 2.08b	59.56 ± 7.44a	16.41 ± 2.20c

[a,b,c]Mean within the same row with different superscript letters, were different ($P < 0.05$)* Level of Significance ($P < 0.05$). PCV—Packed cell volume, RBC—Red blood cell count, Hb—Hemoglobin, WBC—White blood cell, NEU—Neutrophil, LYM—Lymphocyte, EOS—Eosinophil, MON—Monocytes, MCV—Mean Corpuscular Volume, MCH—Mean Corpuscular Haemoglobin, MCHC—Mean Corpuscular Haemoglobin Concentration, PLT—Platelet.

and backcross respectively. The backcross and Hybrid had WBC values of 27.71 ± 2.01 × 10^3/µl and 31.27 ± 1.79 × 10^3/µl respectively which were higher than the normal range (7 to 20 × 10^3/µl) while the NIP value (18.16 ± 3.01 × 10^3/µl) was within the normal range. The NIP and Hybrid had low Neutrophil values of 18.75% ± 1.77% and 12.04% ± 1.87% respectively. It was observed (**Table 1**) that all the red blood parameters were significant ($P < 0.05$) with the exception of RBC and MCHC. NIP had higher values than the Hybrid and Backcross with the exception of RBC where the Hybrid had the highest value of 5.31(10^6/ul). The effect of genotype on Platelet value was significant ($P < 0.05$).

The effect of sex on the haematological parameters of NIP, Hybrid and Backcross is presented in **Table 2**. The means of Red Blood Count traits of male and female are as follow respectively: PCV (32.09% ± 0.83%, 37.23% ± 1.51%); Hb (10.70 ± 0.28 (g/dl), 12.38 ± 0.51 (g/dl)); RBC (4.72 ± 2.76 ($\times10^6$/ul), 4.85 ± 2.12 ($\times10^6$/ul)); MCV (75.63 ± 0.01 fl, 80.35 ± 0.00 fl); MCHC (33.33 ± 0.00 (g/dl), 33.26 ± 0.00 (g/dl)) and MCH (25.21 ± 0.00 pg, 26.73 ± 0.00 pg). The means of Leaukocyte traits of NIP, hybrid and BC are respectively as follows: WBC (24.16 ± 7.68 ($\times10^3$/µl), 25.50 ± 7.28 ($\times10^3$/µl)); NEU (21.51% ± 2.21%, 19.90% ± 2.55%); LYM (75.15% ± 2.26%, 77.87% ± 2.66%); MONO (1.27% ± 0.15%, 1.55% ± 0.22%) and EOS (2.12% ± 0.30%, 1.07% ± 0.24%). The means of Platelet trait of NIP, hybrid and BC are respectively as follow: PLT (33.96 ± 3.53 ($\times10^4$/ul), 43.82 ± 6.14 ($\times10^4$/ul)). The effect of sex was non-significant ($P > 0.05$) in all the white blood parameters with the exception of Eosinophil (2.12% ± 0.30%). Among the red blood parameters, the effect of sex was observed to be significant ($p < 0.05$) in only PCV, Hb and MCV while the RBC, MCHC and MCH were not significant ($P > 0.05$). The values obtained for MCV and MCH, ranging from 68.23 - 87.45 fl, were greater than the normal range (50 - 68 fl) for pigs. It was only the Hybrid that it's MCV (68.23 fl) is within the normal range while NIP (87.45 fl) and Backcross (74.52 fl) values were greater. The Platelet value was significant ($P < 0.05$) with sex.

The Pearson correlation tables for haematological traits of Hybrid, NIP and backcross respectively are presented in **Tables 3-5**. There was high positive correlation (1.00) between Packed Cell Volume and haemoglobin in all the three breeds; and there was negative correlation (−0.99, −0.97 and −0.96) between Neutrophil and the Lymphocyte of Hybrid, Backcross and NIP respectively. This negative correlation between the Neutrophil and Lymphocyte mean that increase in Neutrophil will cause decrease in Lymphocyte.

Table 2. Effect of sex on the haematological traits of NIP, hybrid and backcross.

Variables		Mean	
		Male (33)	Female (31)
Red blood cell count	*PCV (%)	32.09 ± 0.83b	37.23 ± 1.51a
	*Hb	(g/dl)	10.70 ± 0.28b
	RBC ($\times 10^6$/ul)	4.72 ± 2.76	4.85 ± 2.12
	*MCV (fl)	75.63 ± 0.01b	80.35 ± 0.00a
	MCHC (g/dl)	33.33 ± 0.00	33.26 ± 0.00
	MCH (pg)	25.21 ± 0.00	26.73 ± 0.00
White blood count	WBC ($\times 10^3$/µl)	24.16 ± 7.68	25.50 ± 7.28
	NEU (%)	21.51 ± 2.21	19.90 ± 2.55
	LYM (%)	75.15 ± 2.26	77.87 ± 2.66
	MON (%)	1.27 ± 0.15	1.55 ± 0.22
	*EOS (%)	2.12 ± 0.30a	1.07 ± 0.24b
Platelets	*PLT ($\times 10^4$/ul)	33.96 ± 3.53b	43.82

[a,b]Mean within the same row with different superscript letters, were different (P < 0.05)* Level of significance (P < 0.05). PCV—Packed cell volume, RBC—Red blood cell count, Hb—Hemoglobin, Leu—Leucocyte, NEU—Neutrophil, LYM—Lymphocyte, EOS—Eosinophil, MON—Monocytes, MCV—Mean Corpuscular Volume, MCH—Mean Corpuscular Haemoglobin, MCHC— Mean Corpuscular Haemoglobin Concentration, PLT—Platelet.

Table 3. Pearson correlation coefficients for haematological traits of NIP.

	PCV	HB	RBC	MCV	MCHC	MCH	WBC	NEU	LYM	MONO	EOSI	PLT
PCV	1.00	1.00	***0.55**	0.25	−0.01	0.25	−0.23	−0.10	0.13	0.12	−0.30	0.08
HB		1.00	***0.55**	0.24	0.06	0.24	−0.23	−0.11	0.15	0.13	−0.29	0.08
RBC			1.00	***−0.64**	0.07	***−0.64**	***−0.43**	−0.34	0.30	0.04	0.20	0.08
MCV				1.00	−0.08	1.00	0.34	0.26	−0.20	0.14	-	0.38
MCHC					1.00	−0.03	−0.03	−0.20	0.19	0.12	−0.03	0.07
MCH						1.00	0.34	0.25	−0.19	0.15	−0.38	0.02
WBC							1.00	***0.61**	***0.56**	−0.18	0.17	−0.36
NEU								1.00	−0.96	−0.23	−0.13	***0.46**
LYM									1.00	0.15	0.09	***0.50**
MON										1.00	0.25	0.16
EOSI											1.00	0.03
PLT												1.00

Level of significance P < 0.05. PCV—Packed cell volume, RBC—Red blood cell count, Hb—Hemoglobin, WBC—White blood cell, NEU—Neutrophil, LYM—Lymphocyte, EOS—Eosinophil, MON—Monocytes, MCV—Mean Corpuscular Volume, MCH—Mean Corpuscular Haemoglobin, MCHC—Mean Corpuscular Haemoglobin Concentration, PLT—Platelet.

3.3. ASFV Amplification for NIP, Hybrid and Backcross

The 278 bp of ASFV observed in NIP can be corroborated with the findings of Luther *et al.* (2007) [9] and Luther *et al.* (2008) [13], where the same virus band was observed in Bushpigs and Warthogs tested at National Veterinary Research Institute (NVRI), Jos. The presence of ASFV in NIP for long period without any clinical symptom or death was explained by Adeoye and Adebambo (2010) [14] and Oluwole and Omitogun (2013) [10] by the ability of the pigs to trap ASFV within their tissues by the activities of macrophages that eat up the pathogens and infected tissues so that other parts and their mast cells in the tissues are not affected.

Table 4. Pearson correlation coefficient for haematological traits of hybrid.

	PCV	HB	RBC	MCV	MCHC	MCH	WBC	NEU	LYM	MONO	EOSI	PLT
PCV	1.00	1.00	0.04	***0.57**	0.16	***0.56**	0.19	−0.13	0.08	0.18	0.21	0.15
HB		1.00	0.03	***0.56**	0.19	***0.56**	0.19	−0.12	0.08	0.18	0.21	0.14
RBC			1.00	−0.73	−0.20	−0.73	−0.03	−0.20	0.21	0.20	0.30	0.19
MCV				1.00	0.21	1.00	0.10	0.18	−0.22	0.04	0.37	0.08
MCHC					1.00	0.22	0.10	0.12	−0.12	0.10	−0.03	−0.36
MCH						1.00	0.11	0.18	−0.22	0.04	0.37	0.07
WBC							1.00	−0.25	0.25	0.22	−0.22	0.01
NEU								1.00	−0.99	−0.15	0.40	−0.01
LYM									1.00	0.05	***−0.50**	0.01
MON										1.00	0.01	0.33
EOSI											1.00	−0.27
PLT												1.00

*Level of significance P < 0.05. PCV—Packed cell volume, RBC—Red blood cell count, Hb—Hemoglobin, Leu—Leucocyte, NEU—Neutrophil, LYM—Lymphocyte, EOS—Eosinophil, MON—Monocytes, MCV—Mean Corpuscular Volume, MCH—Mean Corpuscular Haemoglobin, MCHC—Mean Corpuscular Haemoglobin Concentration, PLT—Platelet.

Table 5. Pearson correlation coefficients for haematological traits of backcross.

	PCV	HB	RBC	MCV	MCHC	MCH	WBC	NEU	LYM	MONO	EOSI	PLT
PCV	1.00	1.00	0.45	−0.01	−0.23	−0.01	−0.07	−0.06	−0.08	***0.61**	0.26	0.20
HB		1.00	0.45	−0.00	−0.02	***0.00**	−0.07	−0.05	−0.10	***0.62**	0.27	0.20
RBC			1.00	***−0.84**	−0.14	***−0.84**	0.11	0.14	−0.17	0.06	0.35	−0.09
MCV				1.00	0.12	1.00	−0.16	0.12	0.06	0.29	−0.12	0.19
MCHC					1.00	0.12	−0.04	0.27	−0.29	0.10	0.24	0.09
MCH						1.00	−0.16	−0.12	0.05	0.29	−0.12	0.19
WBC							1.00	−0.17	0.25	−0.24	−0.16	0.10
NEU								1.00	−0.97	−0.09	***0.65**	−0.07
LYM									1.00	−0.07	***−0.66**	0.03
MON										1.00	0.21	0.43
EOSI											1.00	−0.01
PLT												1.00

Level of significance P < 0.05. PCV—Packed cell volume, RBC—Red blood cell count, Hb—Hemoglobin, WBC—White blood cell, NEU—Neutrophil, LYM—Lymphocyte, EOS—Eosinophil, MON—Monocytes, MCV—Mean Corpuscular Volume, MCH—Mean Corpuscular Haemoglobin, MCHC—Mean Corpuscular Haemoglobin Concentration, PLT—Platelet.

The Hybrid and backcross also showed the presence of ASFV. This result was corroborated with the findings of Adeoye and Adebambo (2010) [14], where serological tests showed the presence of ASFV in crossbreds. The hybrid and the backcross showing the presence of ASFV was not surprising as ASFV were present in the NIP parent. This observation in hybrid and backcross was reported in their work where serological tests were carried out on ASF outbreak survivors, their offspring and F_2 showed a decline in antibody levels against ASF from 100% to 18.79%. This phenomenon was explained as the ability of engulfed ASFV to be broken down easily and effectively by macrophages leading to decreasing circulation of ASFV in the blood and other tissues. Thus, the amount of shed ASFV particles observed in urine and feaces was seen to drop significantly in the offspring. This observation was also corroborated by Olugasa *et al.* (2007) [15] who also reported that the level of infection in the serum dropped from parents with 96.8% to 13.8% in their offspring.

The ability of the virus to persist in one host while killing another genetically related host was established by Palgrave *et al.* (2011) [16] where a particular sequence found in warthog and bush pigs was absent in domestic pigs.

3.4. Leukocyte Traits in NIP, Hybrid and Backcross

The White Blood Cell (WBC) count is a powerful indicator for infectious and inflammatory disease, leukemia, lymphoma and bone marrow disorders. WBC was found to be higher in backcross compared with NIP and highest in hybrid (**Table 1**). WBC was high in Hybrid because it increased to fight against the virus. Thus, the Leukocyte traits obtained from this study were indications of NIP and backcross been exposed to ASFV and the susceptibility of hybrid to the virus. WBC of NIP ($18.16 \pm 3.01 \times 10^3/\mu l$) was within the normal range (7 to $20 \times 10^3/\mu l$) for domestic pigs while WBC of hybrid and backcross values of $31.27 \pm 1.79 \times 10^3/\mu l$ and $27.71 \pm 2.01 \times 10^3/\mu l$ were higher than the normal range (7 to $20 \times 10^3/\mu l$) (**Table A1** in **Appendix**). This deviation from normal range for domestic pig was also observed to be in agreement with Karalyan (2012) [17], Anderson (1998) [18] and Eze *et al*. (2010) [19] where the WBC ($25.92 \pm 8.08 \times 10^3/\mu l$) of the pigs from South-East of Nigeria were higher than the normal or physiological values (7 to $20 \times 10^3/\mu l$). The observed increase in WBC of Hybrid and backcross was adduced to a probable virus inducement of mass-scale mortality of lymphocytes and neutrophils causing the presence of additional nucleus as reported by Karalyan (2012) [17] and Anderson (1998) [18]. Karalyan (2012) [19] also reported that ASFV infection leads to serious changes in composition of WBC. Stress effects induced due to the presence of pathogen was also presented as a possible cause of increase in WBC [17] [18]. The same trend as observed in WBC was observed in neutrophil and Eosinophil of male and female pigs (**Table 2**). Scientists reported that the neutrophil percentage increased during stress [20]. The significance of increase in Eosinophil of females (1.07%) compared to 2.12% in males in **Table 2** might be due to stress that may be caused by the ASF diseases. Addah *et al*. (2007) [21] reported that high Eosinophil is responsible for the stress, and diseases [22].

3.5. Red Blood Cell Count in NIP, Hybrid and Backcross

Red blood cell parameters are important innate immune cells in blood circulation and they can recognize antigen, kill antigen, they are also involved in immune regulation and have a complete self-regulation system. The RBC of hybrid, NIP and BC were not significant varied from each other from the study. This finding was contrary to findings of De *et al*. (2013) [23] where the PCV values of local pigs of India were significantly different from that of the exotic pigs. In this study, values for RBC, PCV and Hb were observed to be within normal range. Karalyan *et al.* (2012) [17] suggested that ASFV directly or indirectly influenced the haematopoietic homeostasis through activation or impaired haematopoiesis [17] [24]. Also Wardley and Wilkinson (1977) [25] and Quintero *et al.* (1986) [26] reported that ASFV is associated with RBC. Karalyan *et al*. (2014) [19] reported that from the beginning of infection, juvenile forms of RBC, such as the largest cells were observed in the peripheral blood of infected pigs. Among the erythroid precursors, up to 60% of all cells were binucleated, which indicates that acute ASFV infection is accompanied by the emergence of pathological forms of RBC.

The insignificant effect of sex on MHCH and MCH is also corroborated [27] [28].

3.6. Platelet Count in NIP, Hybrid and Backcross

The significant effect of breed on platelet trait was in line with findings of De *et al.* (2013) [23] where the variation between the local breed from India and exotic pigs were significant. Platelet trait in this study for NIP and Hybrid were within the normal range (but that of Hybrid was higher than that of NIP) while the backcross value was lower than the normal range for the domestic pigs. Platelet trait is one of the inflammation signals. Gomez-Vilamandos *et al.* (1996) [24] reported that platelets assist in dissemination of ASFV within infected pig body particularly in sub-acute infection while Anderson (1998) [18] reported that platelet count decreases during acute infections of ASF.

4. Conclusions

In conclusion, ASFV was present in NIP, hybrid and the backcross. The inflammation signal (white blood count, red blood count and platelet count) in hybrid was higher than that of NIP and backcross. ASF in West African

countries especially Nigeria, has become endemic because of the continuous occurring of the intermittent infections. This disease causes large fatalities in pigs and consequently significant loss of income and employment opportunities [29]. It has an overall effect on the pig industry worldwide, because it limits opportunities to explore external markets and increase rapidity/possibilities of inter-continental contamination.

When the presence of the virus is identified, the focus should be on environmental risks and biosecurity in pig. Fashina [30] reported that 306 pig farms and 91% of the 31,916 affected pigs succumbed to the disease in Ibadan (2001), South west Nigeria. Compulsory slaughtering of infected animals and animals at risk, without any compensation to the owners, has dramatic effect on the piggery farmers. The threat of losing their animals with no recompense, drives pig-keepers to do two things to cut their losses, If possible, they will move their animals away from the infected areas, maybe to relatives in neighboring states or countries. In this part of Africa, where sociocultural ties exist regardless of national boundaries, it is relatively easy to move animals over the border to kinsfolk, thereby causing the spread of the disease.

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Appendix

Reference Values for Laboratory Animals (Research Animal Resources, University of Minnesota, 2013).

Table A1. Normal haematology values.

	Dog	Cat	Rhesus	Baboon	**Swine (PIG)**	Sheep	Cow	Rabbit	Guinea Pig	Hamster	Rat	Mouse	Gerbil
PCV (%)	29 - 55	25 - 41	26 - 48	33 - 43	**32 - 50**	24 - 45	24 - 48	30 - 50	37 - 48	40 - 61	36 - 54	39 - 49	43 - 60
Hgb (g/dl)	14.2 - 19.2	14.2 - 19.2	8.8 - 16.5	10.9 - 14.3	**10 - 16**	8 - 16	8 - 15	10 - 15	11 - 15	10 - 18	11 - 19.2	10.2 - 16.6	12.6 - 16.2
MCV (fl)	65 - 80	65 - 80	72 - 86	71.2 - 82.8	**50 - 68**	23 - 48	40 - 60		78 - 95	67 - 77	48 - 70	41 - 49	
MCH (pg)	12.2 - 25.4	12.2 - 25.4	18.5 - 36.6	23.5 - 27.1	**17 - 23**	8 - 12	11 - 17						
MCHC (g/dl)	32 - 36	32 - 36	25.6 - 40.2	31.6 - 34.2	**30 - 36**	31 - 38	30 - 36		27 - 37	30 - 34	40		
WBC (×1000)	5.9 - 16.6	3.8 - 19	7 - 13	3.8 - 15.5	**7 - 20**	4 - 12	4 - 12	7 - 13	4.5 - 11	5 - 8.9	6 - 18	6 - 15	7 - 15
Diff. (%)													
segs	51 - 84	34 - 84	20 - 56	32 - 90	**28 - 50**	20 - 40	20 - 40	20 - 60	28 - 44	17 - 30	10 - 30	10 - 40	5 - 34
bands	0 - 4	0 - 1	0 - 6	0 - 1	**0 - 10**	0 - 2	0 - 4						
lymphs	8 - 38	7 - 60	40 - 76	9 - 63	**40 - 60**	40 - 70	40 - 70	40 - 80	39 - 72	50 - 81	65 - 85	55 - 95	60 - 95
monos	1 - 9	0 - 5	0 - 11	0 - 5	**2 - 10**	0 - 6	1 - 6	1 - 4	2 - 6	0 - 3	0 - 5	1 - 4	0 - 3
eos	0 - 9	0 - 12	0 - 14	0 - 3	**0 - 10**	0 - 10	0 - 4	0 - 4	0 - 5	0 - 4	0 - 6	0 - 4	0 - 4
basos	0 - 1	0 - 2	0 - 6	0 - 1	**0 - 2**	0 - 3	0 - 2	1 - 7	0 - 3	0 - 1	0 - 1	0 - 1	0 - 1
plat (×1000)	160 - 525	160 - 660	109 - 597	151 - 481	**120 - 720**	100 - 800	50 - 750	125 - 270	250 - 850	200 - 500	500 - 1300	160 - 410	400 - 600
Fibrinogen (mg/dl)	200 - 400		118 - 214	100 - 500	**100 - 500**	300 - 700		263 - 572	200 - 300				

Neutrophil = 45% for swine.

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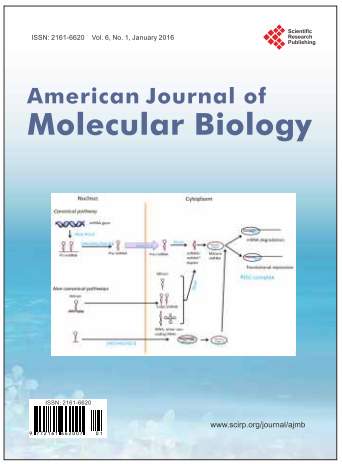

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