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IMMUNOHISTOCHEMICAL STUDY OF PTEN EXPRESSION IN VARIOUS LESIONS OF ATHEROSCLEROSIS

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ABSTRACT

Atherosclerosis is one of the major causes of ischaemic heart disease which in turn leads to millions of deaths annually throughout the world. Several theories have been proposed to explain the process of atherogenesis and plaque formation. Molecular mechanisms are recently gaining importance and particularly the role of PTEN is being studied widely. In this study, we evaluated the expression of PTEN by immuno histochemistry in the various atherosclerotic lesions to elucidate the possible role of PTEN in the pathogenesis of the disease. Immuno histochemistry for PTEN showed varied results in the different types of atherosclerotic lesions and the different grades of the disease. We have also analyzed the previous studies extensively and proposed future areas of research in this aspect.

KEYWORDS: **PTEN, atherosclerosis, cholesterol, macrophages, endothelial injury, lipids, foam cells**

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INTRODUCTION

Ischaemic heart disease is the single cause of death worldwide, leading to roughly 7.4 million deaths annually.Atherosclerosis, the most common underlying pathologic process leading to cardiovascular morbidity and mortality is a multifactorial disease in which hypertension, diabetes,hyperlipidemia and other risk factors are believed to play a role[1]. Atherosclerosis is a systemic disease of the blood vessel wall which is a slow, complex process characterized by accumulation of lipids and thickening of the inner layer of the arterial wall, which, in its earliest form, appears to be present even at the fetal stage, and evolves slowly over decades[3]. This may cause clinical symptoms due to chronic narrowing of the lumen or abrupt plaque erosion or rupture with ensuing thrombus formation.The diagnosis of most ischaemic heart diseases is made on clinical grounds, but there are many ancillary techniques such as biomarkers that have applications in several areas such as screening, diagnosis, prognostication, prediction of recurrences and monitoring of therapy. Among various biomarkers, PTEN is an evolving novel marker whose relative proportions and its expression in various stages of a plaque are being evaluated.

MATERIALS AND METHODS

The study material comprised of 25 cases of atherosclerosis of aorta diagnosed at autopsy. Gross examination of these showed a wide range of lesions ranging from fatty streak, fibrous plaque to complicated plaque. Lesions were defined as fatty streaks if they were flat, white to yellow dots or patches, as fibrous plaques if they were white to yellow, firm elevated lesions, and as complicated plaques if the lesions showed ulceration or calcification. The spectrum of cases taken for the study were: Fatty streak – 14; Atheromatous plaque – 6; Complicated laque – 5 These specimens were obtained from autopsy from patients of various ages who died of different causes at Government Royapettah Hospital, Chennai. Hematoxylin and eosin stain was done to diagnose the various lesions. The relative distribution of PTEN in various stages of atherosclerotic plaque was studied by immunohistochemical methods, using formalin-fixed, paraffin-embedded sections.

PRINCIPLE OF IMMUNOHISTO CHEMISTRY EMPLOYED

Micropolymer technique (ImmPRESS Universal reagent)

The Imm PRESS Universal reagent contains a micropolymer of a very active peroxidase coupled with a mixture of our affinity-purified anti-mouse IgG and anti-rabbit IgG secondary antibodies. These enzyme “micropolymers” avoid the use of large dextrans and other macromolecules as a backbone. This is based on a new method of polymerizing enzymes and attaching these polymers to antibodies, thus allowing a higher density of enzymes to access a target with minimal interference, resulting in reduction of the number of steps in the protocol, an increase in signal intensity and significantly less background staining. The entire antigen-antibody-enzyme complex is made visible by the addition of a chromogenic substrate, DAB (diaminobenzidine tetrahydrochloride) by localizing peroxidase.Positivity was interpreted when the nuclei of the cells took up a brown color.

RESULTS

PTEN showed nuclear positivity of the vascular endothelial cells, sub endothelial cells and also of the vascular smooth muscle cells which had migrated to the intima. The endothelial cells of the vessels of the vasa-vasorum served as the internal control. In the normal aorta, PTEN showed marked endothelial and sub endothelial positivity (Figure 3). Out of 14 cases of fatty streak, 50% of the cases also showed marked endothelial and sub endothelial positivity (Figure 4). Out of 6 cases which showed an intermediate lesion to a well formed atheromatous plaque, reduced endothelial, sub endothelial and vascular smooth muscle positivity was observed in about 83% cases (Figure 5). The complicated lesions which had calcification, which formed about 5 cases in our study, did not show any positivity in the

lesions (Figure 6).However about 36% of the fatty streak showed reduced intensity of staining in the endothelial and sub endothelial cells and about 14% of the cases showed negative staining. (Table 1& 2.)In each grade of atherosclerosis, PTEN was expressed at different levels. Among 2 cases of Grade I lesion, 50% of them showed both increased intensity and reduced intensity ofstaining.Among 12 cases of Grade II lesion, 50% showed increased intensity of staining, 33% showed decreased intensity of staining and 17% showed no expression of PTEN.Among 3 cases of Grade III lesion, 67% showed reduced staining for PTEN while 33% showed a negative staining and in 3 cases of Grade IV lesion, 67% showed reduced staining and 33% showed increased expression of PTEN.Among 2 cases of Grade V and 3 cases of Grade VI lesions, 100% of both grades showed negative staining for PTEN expression (Table 3).

Table 1
PTEN expression in atherosclerosis

Sl.No	Block No.	Age/ Sex	Lesion	Grade	Staining Pattern			Comments
					Endothelial cells	Sub-endothelial cells	VSMC	
1.	58/09	36/M	Fatty streak	II	+++	+++	-	Well expressed
2.	47/08	48/M	Atheromatous plaque	III	++	++	+	Less expressed
3.	76/09	33/M	Fatty streak	II	+++	+++	-	Well expressed
4.	56/09	28/M	Fatty streak	I	+	+	-	Less expressed
5.	36/08	42/M	Fatty streak	II	++++	++++	-	Well expressed
6.	48/09	60/M	Atheromatous plaque	IV	++	++	+	Less expressed
7.	49/09	32/M	Fatty streak	II	+++	++	-	Less expressed
8.	50/09	48/M	Atheromatous plaque	IV	++	+++	+++	Well expressed
9.	54/08	55/M	Complicated plaque	VI	-	-	+	Negative staining
10.	56/08	37/M	Atheromatous plaque	III	-	+	-	Negative staining
11.	48/08	34/M	Fatty streak	II	+	+	-	Less expressed
12.	71/08	68/M	Complicated plaque	VI	-	-	-	Negative staining
13.	38/08	40/M	Fatty streak	II	+	+	-	Less expressed
14.	74/08	52/M	Complicated plaque	V	-	+	+	Negative staining
15.	33/08	50/M	Complicated plaque	VI	-	-	-	Negative staining
16.	37/08	38/M	Fatty streak	II	-	-	-	Negative staining
17.	64/08	36/M	Fatty streak	II	+	+	-	Negative staining
18.	50/08	29/M	Fatty streak	II	+	+	-	Less expressed
19.	57/08	30/M	Fatty streak	II	+++	++	-	Well expressed
20.	61/08	45/M	Complicated plaque	V	-	-	-	Negative staining
21.	65/08	48/F	Atheromatous plaque	IV	++	+	+	Less expressed
22.	62/08	37/M	Atheromatous plaque	III	+	++	+	Less expressed
23.	77/08	20/M	Fatty streak	II	+++	+++	-	Well expressed
24.	53/08	27/M	Fatty streak	I	++++	++++	-	Well expressed
	72/08	32/M	Fatty streak	II	+++	++	-	Well expressed

Table 2
PTEN expression in different lesions of atherosclerosis

Lesion	Negative staining	Less expressed	Well expressed	Total cases
Fatty streak	2 (14%)	5 (36%)	7 (50%)	14
Atheromatous plaque	1 (17%)	4 (66%)	1 (17%)	6
Complicated plaque	5 (100%)	-	-	5

Figure 1
PTEN expression based on the type of lesion

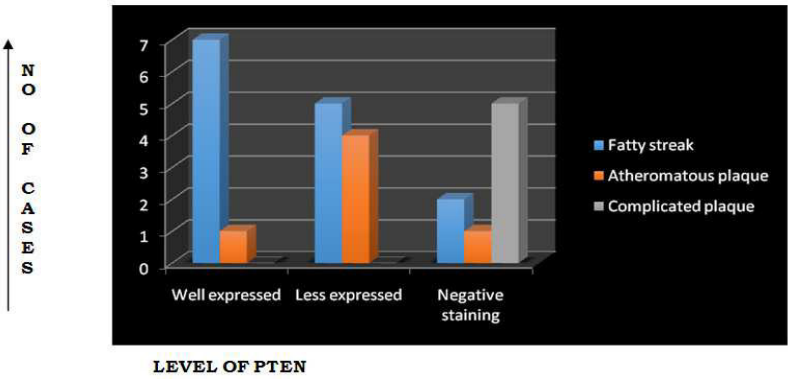


Table 3
PTEN expression in different grades of atherosclerosis

Grade	Negative staining	Less expressed	Well expressed	Total cases
I	-	1 (50%)	1 (50%)	2
II	2 (17%)	4 (33%)	6 (50%)	12
III	1 (33%)	2 (67%)	-	3
IV	-	2 (67%)	1 (33%)	3
V	2 (100%)	-	-	2
VI	3 (100%)	-	-	3

Figure 2
PTEN expression based on the grade of the lesion

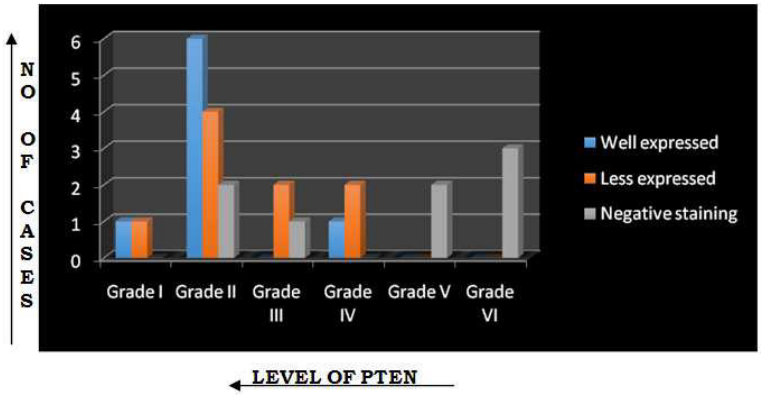


Figure 3
Normal aorta: PTEN positivity in endothelium and sub-endothelial tissue X400

Figure 4
Marked endothelial PTEN positivity in fatty streak X400

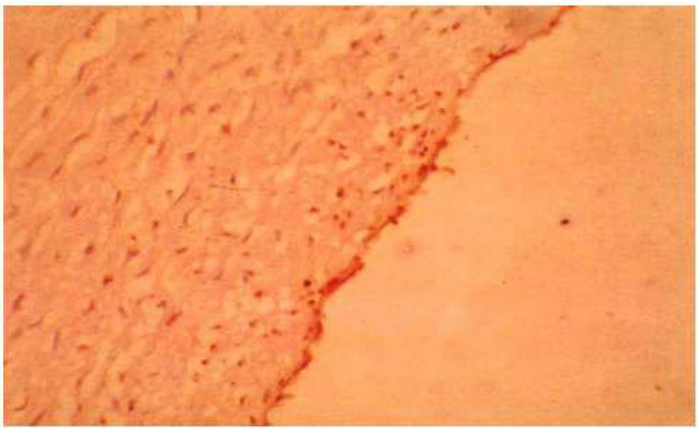


Figure 5
Reduced endothelial & subendothelial PTEN positivity in atheromatous plaque X400

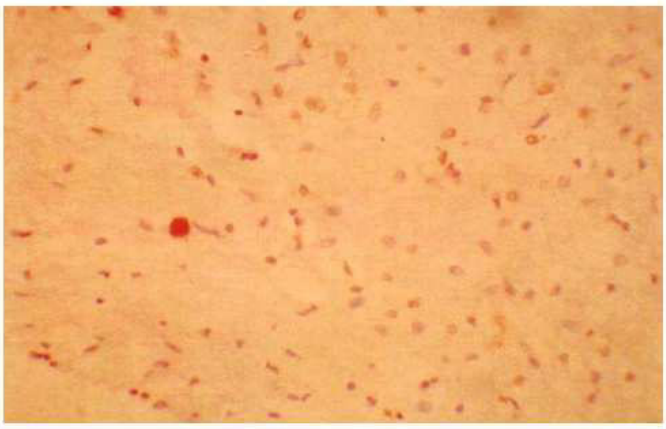
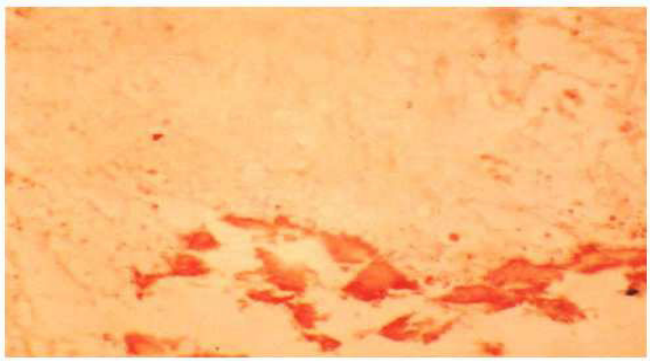


Figure 6
Negative PTEN staining in a complicated plaque X400



DISCUSSION

Atherosclerosis (Greek 'athero' = gruel/paste; 'sclerosis' = hardening) was coined by Marchand in 1904, and later defined by a study group of WHO as 'a variable combination of changes of the intima of arteries consisting of focal accumulation of lipids, complex carbohydrates, blood and blood products, fibrous tissue and calcium deposits associated with medial changes'[2].Atherogenesis is a complex

process characterized by lipid retention, proteolytic injury and a chronic inflammatory response.The lesions of atherosclerosis take different forms depending upon their anatomic site; the age, genetic constitution, and physiologic status of the affected individual; and the risk factors to which each individual has been exposed. Examination of the lesions reveal that each lesion contains the elements of an inflammatory response together with varying levels of fibroproliferative response[3].It is now evident that inflammatory/immunologic processes play a major role in the development of atherosclerosis[4].The American Heart Association classification divides atherosclerotic lesions into six types, beginning with isolated foam cells ("fatty dots"), through stages of fatty streaks, atheromas, and fibroatheromas, to the complicated lesions[5].A recent Bethesda Conference proposed a classification scheme according to the strength of evidence that risk factor intervention favorably affects outcome [6].

Morphology

In atherosclerotic arteries the major changes are in the intima. These are considered to be the results of intimal injury. Characteristic lesions have been identified, namely fatty streaks, fibrous plaquesand the complicated lesion. The fatty streakis characterized by a focal accumulation of smooth muscle cells which contains lipid and is surrounded by lipid deposits. The lipids found within the smooth muscle cells are cholesterol and cholesteryl esters. With the accumulation of extracellular lipid in the intima, a transitional plaque is formed and with smooth muscle proliferation surrounding the lipid producing increasing amounts of collagen,forms an advanced or fibrolipid plaque. The final transition is to an unstable plaque complicated by thrombosis. If there is no lipid core but a preponderance of smooth muscle cells, we have a solid fibrousplaque. A further variant contains many foam cells and is described as gelatinous. The main components of atherosclerotic plaques are connective tissue and extracellular matrix with smooth muscle cells forming a fibrous cap, intracellular and extracellular lipids with inflammatory cells such as monocyte-derived macrophages and T-Lymphocytes[7] forming the core.

Molecular pathogenesis of atherosclerosis

Four important mechanisms are thought to play a role.

- Chronic endothelial injury results in endothelial dysfunction, releasing ET-1, a potent vasoconstrictor that increases leukocyte-endothelial interactions and platelet-endothelial interactions[8]yielding increased permeability and leucocyte adhesion by stimulating the expression of ICAM and VCAM that mobilize inflammatory cells towards the vascular lumen.
- Retention of LDL with subsequent oxidation giving rise to oxidatively modified LDL (oxLDL) plays a pivotal role in attracting more macrophages to the site of the lesion.
- Monocytes attracted to lesion-prone sites by cell-adhesion molecules migrate through the endothelial layer into the intima where, under the influence of factors such as monocyte colony-stimulating factor (M-CSF), they take up oxidized LDL (oxLDL) to become macrophages[9], producing various chemokines, including monocyte chemotactic protein-1 (MCP-1) recruiting more leukocytes. Reactive oxygen species (ROS) production is increased after the stimulation of endothelial cells by TNF-α and other cytokines both intracellularly and extracellularly causing lipid peroxidation, endothelial injury and DNA damage[10].
- An important step in the migration and proliferation of VSMC is believed to be the secretion of matrix metalloproteinase's (MMP), especially MMP9[11] in response to the cytokines such as IL-1 and TNF-α stimulating the local production of PDGF and FGF, which have a pivotal role in plaque formation and complications.

PTEN: ***The tumour suppressor gene on chromosome 10q23***

In 1997, Li et al. identified a putative tumor suppressor on chromosome 10q23. Sequence analysis of the 403 amino acid open reading frame revealed a protein tyrosine phosphate domain and a large region of homology to chicken tensin and bovine auxilin, the gene was, therefore, designated PTEN; for phosphatase and tensin homologue deleted on chromosome 10[12].Steck et al. independently reported the identification of the same candidate tumor suppressor gene on 10q23, which was termed as MMAC1 for Mutated in Multiple Advanced

Cancers[13].Finally, in a search for novel human protein tyrosine phosphatases, a gene termed TEP1 (TGF-β regulated and Epithelial cell enriched Phosphatase) was identified[14] which was proved to be identical to PTEN.

Structure of PTEN

The initial study of PTEN showed that it was a 403-aminoacid protein with a relative molecular mass of 4.7 KD,that contained an amino-terminal region with homology to auxilin and tensin and a protein phosphatase domain.Solving the crystal structure of PTEN revealed a phosphatase domain that was similar to the protein phosphatases but had an enlarged active site, necessary for the accommodation of a phosphoinositide substrate that accounted for its ability to dephosphorylate lipid substrates[15] anda C2 domain that had affinity for phospholipid membranes in vitro containing two PEST sequences and a PDZ motif in the carboxy-terminal (C-terminal region) whose function is essential in PTEN's tumour suppressor function[16]. The function of PEST sequences is to target proteins with short intracellular half-life for protein degradation. The C-terminal region appears to be important in PTEN stability and enzymatic activity.Removal of PDZ domain from PTEN greatly reduced its ability to inhibit Akt activity, which points to the potential importance of the interaction of PTEN and membrane-associated guanylate kinase inverted (MAGI) proteins in facilitating PTEN's function as a phosphatase[17].

Molecular basis of PTEN function

The function of PTEN is as a multifunctional phosphatase that removes phosphorylates from tyrosine and serine residues, as well as from phosphatidylinositols, especially phosphatidylinositol(3,4,5) triphosphate. The most biochemical consequence of PTEN is to disarm the PI3'-kinase / AKT pathway. Specifically, mutations in PTEN are associated with an increase in AKT activity, which is a pivotal member of a pathway that induces cell survival and motility. It is thought that AKT phosphorylates the bcl-2 proapoptotic homologue, bad, resulting in a block to apoptosis.These actions of PTEN are mediated by antagonism of the phosphatidylinositol 3-kinase (PI3K)-mediated signaling pathway modulating the activity of AKT, and of their downstream substrates such as Gsk3β, $p70^{s6k}$, the bcl-2 family member bad, procaspase 9, 4E-BP1, IKKα, and the members of the Forkhead family of transcripton factors. In cancers that have lost PTEN function, there is an unopposed activity of AKT and their substrates, leading to more rapid growth in cell size and protein translation[18].PTEN has also been shown to inhibit hypoxia and IGF-1 mediation induction of Hypoxia induced factor-1, a factor shown to upregulate genes important for angiogenesis, leading to apoptosis and cell cycle arrest, and downregulate genes required for angiognenesis.In addition to its tumor suppression capability, PTEN is also known to modulate several cell functions including migration, proliferation, invasion and cytoskeletal rearrangement through its phosphatase activity, which downregulates signaling pathways involving focal adhesion kinase (FAK) or Shc. Modulation of FAK/Shc activity by PTEN affects cell migration and adhesion activated by integrins and other tyrosine kinase receptors. PTEN is expressed endogenously and regulates many cell functions in cardiomyocytes, fibroblasts, endothelial cells, and vascular smooth muscle cells (VSMCs), including proliferation, migration, survival/apoptosis, hypertrophy, contractility, metabolism, and mechano-transduction[19].

Role of PTEN in atherosclerosis

Shinichiro Koide et al. (2007) in their research found a rat cuff injury model, that overexpression of PTEN directly inhibited ANG-II induced MCP expression in cultured VSMC's, reducing neointima formation through inhibition of the proinflammatory response involving macrophage invasion and cytokine expression in addition to suppression of cell proliferation and migration and increased apoptosis[20].Jianhua Huang et al. (2005) found that the overexpression of PTEN in VSMC's with a recombinant adenovirus inhibited PDGF induced cellular proliferation, migration and survival, suggesting that the overexpression of PTEN in vivo might disrupt neointimal hyperplasia because the number of medial cells was markedly reduced early after virus infection, and a high percentage of the remaining cells demonstrated apoptotic morphologic features and positive TUNEL

staining[21].Jonathan A.Hata et al. (2005), modulated the PI3K signaling pathway through Akt, with resultant decreasing vascular smooth muscle cell growth and survival, by administering a recombinant, replication-deficient adenovirus directing the expression of wild-type human PTEN which reduced intimal hyperplasia in aortocoronary saphenous vein grafts[22].Increased SMC proliferation of SDF-1 has been implicated in the recruitment of bone marrow derived progenitor cells expressing the SDF-1 receptor CXCR4, which contribute to neointima formation. Thus, vascular accumulation of SDF-1 is critical for targeting CXCR4-positive cells to the site of injury[23]. In view of the above, RaphelA.Nemenoff et al. (2008) inactivated PTEN in VSMC's establishing an autocrine growth loop increasing progenitor cell recruitment through a HIF-1 mediated SDF-1/CXCR4 axis, resulting in vascular remodeling.Paul Deleris et al. (2003) found the expression of 5-phosphatase SHIP-2 (src homology 2 domain-containing inositol phosphatase) which is the primary enzyme for metabolizing phosphotidylinositol bi- and tri-phosphates as well as the 3-phosphatase PTEN which downregulates PI3K signaling in VSMC nuclei forming a major role in the pathogenesis of atherosclerosis[24].Platelet derived growth factor (PDGF) is a potent activator of PI3K which is associated with tissue overgrowth such as atherosclerosis, rheumatoid arthritis and cancer. Yoko Takashi, et al. (2006) found that NHERF (Na^+/H^+ exchanger regulatory factor) proteins recruit PTEN suppressor protein to attenuate PDGF receptor and restrict the activation of PI3K[25].Hyperleptinemia, which often coexists with diabetes and metabolic syndrome, is an independent risk factor for progression of coronary artery disease. Jian Shan et al. (2008) found that a combination therapy with mTOR and PI3K inhibitors, PTEN or LY294002 inhibits leptin induced neointimal hyperplasia[26].Konstantin Tsoyi, et al (2009) used a synthetic tetrahydroisoquinoline alkaloid, CDK712 against lipopolysaccharide treated human umbilical vein endothelial cells which selectively inhibits lipopolysaccharide-induced VCAM-1 expression in vascular endothelial cells by upregulating PTEN playing a pivotal role in the checkpoint of atherosclerosis[27].Molecular studies and other studies such as immunoblotting done by Shinichiro Koide et al (2007. locćit), Jianhua Huang et al (2005. locćit) and Jonathan A. Hata et al (2005. locćit) reveal that PTEN downregulation plays a pivotal role in vascular smooth muscle cell proliferation, neointimal formation, lipid accumulation and other key pathogenic mechanisms in atherosclerosis. These findings correlate well with our study of immunohistochemical expression of PTEN in which significantly reduced immunoexpression was found, as the grade of atherosclerosis progressed from a fatty streak to a complicated plaque.Research by workers such as Raphel A. Nemenoff et al. (2008. locćit), Jian Shan et al. (2008. locćit) and Konstantin Tsoyi et al. (2009. locćit) have proved that PTEN, in future, would be a target for guided chemotherapy and gene therapy in treating potentially life threatening diseases like coronary and cerebral atherosclerosis. Since, present day therapy is focused towards prevention and reduction in the severity of this enigmatic entity and as cure is not offered by the drugs once the atherogenic events have led to the formation of plaque, more studies directed towards the restoration of the affected arteries to their pre-atherosclerotic state are needed and therapy targeted towards increasing the molecular expression of PTEN through various vectors, which are in the experimental stage can pave way for a complete cure of this disease.In this study, we have found PTEN is significantly expressed in the nucleus of endothelial cells of the normal vessels. Though it is also well expressed in fatty streak and the expression in them almost parallels that of normal vessels, it was interesting to observe that there was a reduced to absent staining in a few cases. It is our view that these lesions which showed altered staining patterns could be precursor lesions and might progress to frank atherosclerosis.Histomorphological analysis of various lesions revealed evidence of vascular smooth muscle cell proliferation in the intima of arteries, endothelial proliferation, foam cell formation and calcification. In cases with features pathognomonic of atherosclerosis, there was a progressive reduction in PTEN immunoexpression. Though quantitative assessment of the reduction of expression of PTEN was not done in our study, the

qualitative reduction in the levels of PTEN in these lesions was noted. As not many studies have been performed in the immunohistochemical expression of PTEN, our study gains significance.We feel that immunohistochemical study of PTEN is a more accessible and available method to analyze the prognostic indicators in atherosclerosis. Since the altered expression parallels the increase in the grade of the lesion, PTEN proves to be a useful marker to predict the inherent pathogenesis and behavior of subclinical and early lesions.

CONCLUSION

Atherosclerosis is a constitutionally determined disease of the arteries which begins in the early years of life and follows a variable course thereafter. A thorough understanding of the pathogenesis and interactions between the various implicated factors requires more research. Many attempts have been made at the biochemical level to predict the course of the disease at early stages such as elucidating the varied causes of hyperhomocysteinemia[28] and finding ways to take precautionary measures.PTEN, an extensively studied tumor suppressor gene which has a dual phosphatase activity playsa major role in various tumors and is also recently attributed in atherogenesis. PTEN is believed to act through modulation of the phosphatidylinositol 3-kinase signaling through Akt pathway, altering signals for cell survival, apoptosis and cell migration. In this study, we have tried to correlate the expression of PTEN in various types of atherosclerosis through immuno histochemical methods.From this study, we conclude that PTEN plays a pivotal role in the pathogenesis of atherosclerosis. It is well expressed in the normal arteries and to a similar extent in the fatty streak. But as the type of lesion progresses through an atherosclerotic plaque to a complicated plaque, there is a significant and progressive reduction in the immunohistochemical expression of PTEN in these lesions.Therefore, PTEN proves to be a promising candidate for further studies directed towards characterization of the progression of atherosclerosis.

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