Investigation of Loss of Heterozygosity of *Pten* Gene in Cervical Carcinoma

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ABSTRACT

Phosphatase and tension homolog deleted on chromosome ten (Pten) is a putative tumour suppressor gene located on chromosome band 10q23. Mutations in *Pten* have been identified in numerous human malignancies, including cancers of the brain, endometrium, ovary, and prostate. In this study, we screened 25 female patients suffering from cervical cancer for loss of heterozygosity (LOH) at 10q23, using the microsatellite markers D10S198. Polymerare chain reaction (PCR) products were loaded on to denaturing polyacrylamide gel and electrophoresed.The gel was then visualized by silver staining. All samples, in which two distinct alleles of similar intensity were present in the normal deonyribo-nucleic acid (DNA), were considered to be informative. LOH was scored as positive when a clear reduction of signal intensity (or more than 50%) detected in one of the alleles of the tumor DNA compared with the paired normal DNA. In instances where more than one band was present within each allele, the exact position of each allele was decided by comparing the banding pattern in all samples analyzed with the same marker and selecting the most consistent pattern: the two alleles were identified as two groups of bands of similar number and similar signal intensity.

PCR-based denaturing gradient gel electrophoresis revealed intragenic *Pten* mutations in 32% of the total cervix cancer samples studied (25 samples). LOH analysis was performed on the 25 cervical cancer samples with corresponding germ line DNA available using one microsatellite marker of flanking region of *Pten* (D10S198). Out of 25 samples studied eight pairs were informative at the specific marker. Rest of the samples showed no allelic loss. All samples showing LOH were

subjected to repeat amplification and analysis for confirmation.

Key words: Cervical carcinoma, *Pten* gene, loss of heterozygosity

INTRODUCTION

Cancer is a complex disease occurring as a result of progressive accumulation of genetic and epigenetic changes that enable cells to escape from normal cellular and environmental control (Weinberg, 1996)

The terms "*TSGs*" and "*Oncongenes*" traditionally use to indicate those genes whose alteration leads to tumor initiation and progression, have now been joined by the terms "Gatekeeper and Caretaker" (Kinzler and Vogelstein,1997). The pathways regulating the cell cycle incorporate both Oncogene and TSGs and are frequently deregulated in human cancers and increased genetic instability. Tumor suppressor genes code for anti proliferation signals and proteins that suppress mitosis and cell growth. Generally, tumor suppressors are transcription factors that are activated by cellular stress or DNA damage. Often DNA damage will cause the presence of free-floating genetic material as well as other signs, and will trigger enzymes and pathways, which lead to the activation of TSGs. The functions of such genes is to arrest the progression of the cell cycle in order to carry out DNA repair, preventing mutations from being passed on to daughter cells (Marshall, 1991). A candidate tumor suppressor gene *Pten* (also known as MMAC1 or TEP1) has recently been isolated from chromosome 10q23.3. They are found mutated in several cancer types that display LOH in this region. The *Pten* gene encodes a 403 amino acid protein homologous to some protein phosphatases, and the protein has been shown to possess protein phosphatase activity *in vitro*. It is thought that *Pten* protein dephosphorylates the 3 positions of phosphatidylinositol 3,4,5-triphosphate (PIP3), a well-known intracellular messenger of certain cell-growth stimulator. The molecular mechanisms of *Pten* have been elucidated recently, and it is considered that *Pten* belongs to a class of tumor suppressor genes together with p^{53}, Rb, and APC.

Cervical cancer, a common kind of cancer in women, is a disease in which cancer cells are found in tissue of the cervix. Cancer of the cervix can take many years to develop. Before it does, early changes occur in the cells of the cervix. The name given to these abnormal cells, which are not cancerous but may lead to cancer, is cervical intra-epithelial neoplasia (CIN). Some doctors call these changes precancerous, meaning that the cells have the potential to develop into cancer if left untreated. It is important to know that the vast majority of women with CIN do not develop cancer. CIN may also be referred to as dyskaryosis. The cervix is

the opening of the uterus (womb). The uterus is the hollow, pear-shaped organ where a baby develops. The cervix connects the uterus to the vagina (birth canal) (Sitas *et al.*, 1997; American Cancer Society, 2008). Loss of heterozygosity (LOH) in a cell represents the loss of normal function of one allele of a gene in which the other allele was already inactivated. Loss of heterozygosity can be identified in cancers by noting the presence of heterozygosity at a genetic locus in an organism's germline DNA, and the absence of heterozygosity at that locus in the cancer cells. This is often done using polymorphic markers, such as microsatellites or single nucleotide polymorphisms, for which the two parents contributed different alleles (Bose *et al.*, 1998).

MATERIAL AND METHODS

Tissue samples

The cancerous tissues samples were collected from 25 different patients at Loknayak Jaiprakash Hospital (LNJP), New Delhi. Source of the tissue samples was from the biopsy sample tissues (fresh tissues). The biological material collected were around 5-10mg in PBS. The tissues stored in the PBS were used for DNA isolation. The tissues were stored at -20°C/-70°C or in liquid nitrogen until further analysis. Transportation of the material was carried out using ice buckets.

DNA isolation

DNA was extracted from cervical carcinomic tissue and the corresponding normal tissues. The aim of all DNA preparation method is to obtain unbroken DNA from tissues. The use of DNA for analysis usually requires it to be isolated and purified. To obtain DNA, free from protein, cells are first lysed using detergent and then digested with proteinase K to make deproteinization easier. Protein are then separated from the solution by extracting repeatedly with phenol/chloroform and separates out as an interphase between phenolic and aqueous layer after centrifugation. DNA and RNA get separated in the aqueous layer. DNA is precipitated using ethanol in the presence of sodium acetate. Method for the approximate determination of the concentration of genomic DNA was electrophoresis of 1-2 µl of DNA solution on an ethidium bromide stained 0.8% agarose gel in 1 X TE buffer and intensity was observed.

LOH analysis

To check the allelic loss of *Pten* gene in cervical carcinoma, loss of heterozygosity was performed using a specific marker for that locus. The marker

used for this study was D10S198. Initially, PCR was done using primers specific for the microsatellite marker D10S198. The sequence for forward primer (D10S198- F) is TGAGGGACTCATCTTCTGTT while the sequence for backward primer (D10S198-R) is GTCTGTGATCCCCATGTTAG.

First of all, the PCR mixture was prepared for five samples. Each sample has 25µl in volume. Each of the PCR mixtures contained 2µl genomic DNA, 2µl Taq buffer, 0.1mL of each primer, 0.5mL of dNTPs, 0.5 unit of Taq DNA polymerase and 17.5mL distilled water (Table 1). PCR was carried out over 35 amplification. PCR Conditions are given in (Table 2).

5 to 10µl of each PCR product was loaded on a 2.5% agarose gel and electrophoresed to check the amplification of the desired product using specific ladder of 50 bp to evaluate the amplification of the PCR product at its actual amplification. The gel was visualized under UV transilluminator.

After the amplification, PCR products were resolved on denaturing polyacrylamide gel (100 ml of 6% Polyacrylamide gel) and the electrophoresed samples

Table 1. Pten (Marker-198) RXN- 25 µl for 5 samples

Distilled water	17.5 X 5	87.5
Taq buffer	2.5 X 5	12.5
dNTP	0.5 X 5	2.5
Primer for (D10S198) (Forward)	1.0 X 5	5.0
Primer for(D10S198) (Reverse)	1.0 X 5	5.0
Taq	0.5 X 5	2.5
DNA Template	2.0 X 5	10.0
		Total = 125µl

Table 2. PCR conditions set for amplification of D10S198 microsatellite marker

Initial Denaturation	94°C for 10min
Denaturation	94°C for 30 sec
Annealing	58°C for 1 min
Extension	72°C for 1 min
Final Extension	72°C for 7min
Soaking	04°C
Number of Cycles	35

were stained. For staining developer, 10% Ethanol; 0.7% Nitric Acid; 3% Acetic Acid and 0.2% Silver Nitrate were prepared.

RESULTS

LOH analysis was performed on the 25 cervical cancer samples with corresponding germ line DNA available using one microsatellite marker of flanking region of *Pten* D10S198. Out of 25 samples eight pairs were informative at the specific marker. Rest of the samples showed no allelic loss. All samples showing LOH were subjected to repeat amplification and analysis for confirmation.

DISCUSSION

There is strong circumstantial evidence that allelic loss of *Pten* is found in a large variety of human cancers. The inactivation of *Pten* gene may be due to mutation and LOH or decreasing expression of *Pten* mRNA or protein. Tumorigenesis is the result of a multi step process resulting in genetic alteration that derive the progressive transformation of normal cells into malignant derivatives. Tumor suppressor genes (TSGs) are defined as genetic elements whose loss or mutational inactivation allows cells to acquire a neoplastic phenotype. The concept of multiple genetic events responsible for tumorigenesis was well established. The study of oncogenes and subsequently tumor suppressor genes play an important role in cancer research. Frequent LOH was observed on chromosomes 4p, 6q, 7p, 8q, 10, 11, 12, 13, 16, 17, 18 and 19p. Chromosome 6q includes the sites for the estrogen receptor and the proto-oncogene myb and therefore, has been carefully studied. Frequent loss of heterozygosity (LOH) within genetically defined chromosomal regions is considered and indication of the presence of a putative TSG.

Pten / MMAC (mutated in multiple advanced cancers) have been identified as tumor suppressor gene located at 10q23. A broad spectrum of somatic *Pten* mutations in primary tumors has been reported and includes null mutations, missense mutations, and truncations, which span from the *Pten* promoter, the phosphatase domain in the N-terminus, the C2 domain in the middle, and the C-terminus of *Pten*. Loss of heterozygosity of markers along the long arm of chromosome 10 occurs in cervical cancer. A small study based on Loknayak Jaiprakash Hospital (LNJP) cervix cancer samples, 8 samples out of 25 have shown biallelic structural inactivation occurence either by homozygous deletion at 10q23 or somatic intragenic *Pten* mutation plus loss of the remaining wild type allele. Instead, even in the primary setting, hemizygous deletion at 10q23, encompassing *Pten*, can occur with some frequency (32% of a small number) as an early event.

REFERENCES

American Cancer Society. 2008. Cancer facts and figures. *Atlanta, Ga.*

Bose, S., Wang, S. I., Terry, M. B., Hibshoosh, H. and R. Parsons. 1998. Allelic loss of chromosome 10q23 is associated with tumor progression in breast carcinomas. *Oncogene*, **17**:123-127.

Kinzler, K.W., and B. Vogelstein. 1997. Cancer-susceptibility genes. Gatekeepers and caretakers. *Nature*, **386**: 804-810.

Marshall, C.J. 1991. Tumor suppressor genes. *Cell*, **64**: 313-326.

Sitas, F., Carrara, H., Terblanche, M., and Madhoo, J. 1997. Screening for cancer of the cervix in South Africa. *S Afr Med J.*, **68**: 935-936.

Weingberg, R.A. 1996. How cancer arises. *Scientific American.*, **275**: 62-65.