Clinical Relevance of Cytogenetic Abnormalities in Plasma Cell Myeloma

Role of MSI Testing in Colorectal Carcinoma

PTEN in Prostate Cancer: A Potential Therapeutic Target

ON THE COVER: Paraffin section FISH analysis of plasma cell myeloma
Fluorescence in situ hybridization (FISH) employs DNA probes labeled with fluorescent markers of various colors to detect numerical and structural chromosomal abnormalities in tumor cells. FISH has many applications in diagnostic pathology and is frequently employed in the diagnosis and classification of lymphomas and leukemias. Today, FISH analysis is playing a growing role in the evaluation of another hematolymphoid malignancy — multiple myeloma.

Multiple myeloma is a multi-focal, bone marrow-based neoplasm of plasma cells associated with lytic bone lesions, hypercalcemia, and serum and/or urine monoclonal paraproteins. The disease typically affects the middle-aged to elderly, with an average age at diagnosis of approximately 70 years. Multiple myeloma is typically incurable with an average survival of 3 years and fewer than 10 percent of patients surviving for 10 years after diagnosis.

In approximately half of all multiple myeloma, chromosomal translocations are present that involve the immunoglobulin heavy chain (IGH) locus at chromosome 14q32 and one of several translocation partner genes. Several of these translocations are associated with distinct clinical and/or pathologic features.

The t(4;14)(p16.3;q32), involving IGH and the MMSET and FGFR3 genes on chromosome 4p16.3, is associated with an unfavorable prognosis. In contrast, the t(11;14)(q13;q32), involving IGH and the CCND1 gene at 11q13, does not carry an adverse prognosis but is associated with lymphoplasmacytic morphology that could lead to misdiagnosis as lymphoma.

Identification of these abnormalities clearly can provide useful information in the diagnosis and prognostic assessment of multiple myeloma. Because plasma cells grow poorly in culture, these types of abnormalities are difficult to detect by metaphase cytogenetic studies. FISH analysis offers the ability to detect these abnormalities in non-dividing cells.

FISH analysis of multiple myeloma, however, presents a unique technical challenge. In most cases of multiple myeloma, the number of malignant plasma cells is smaller than the number of admixed normal hematopoietic cells in the bone marrow. The normal nuclei will lack any chromosomal translocations, essentially diluting out the malignant cells and making scoring of FISH results difficult.

Useful FISH analysis of multiple myeloma, therefore, requires a technique to allow scoring of results in plasma cells only. We have countered this problem by developing an assay that employs intact, thin paraffin sections of bone marrow clot and simultaneous immunofluorescence to specifically identify plasma cells.

In this technique, clotted bone marrow is fixed in formalin, and standard thin sections of paraffin-embedded tissue blocks are prepared. The sections are stained with an antibody to CD138, a plasma cell marker. Using a tyramide signal amplification method, a fluorescent marker is linked to the CD138-binding plasma cells. Next, the FISH probes of interest are hybridized to the slide, and results are visualized under a fluorescent microscope. Plasma cells are specifically identified by blue cytoplasmic staining for CD138, and the FISH pattern seen in the nuclei of labeled cells is scored. The nuclei of unlabeled, normal bone marrow elements are not counted.

Several FISH probes currently are available for analysis of multiple myeloma. Cases are initially screened using a two-color, break-apart assay that employs two probes that flank the IGH locus, one labeled with red and one with green. In normal cells, the red and green signals

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By Mary P. Bronner, M.D.

Hereditary non-polyposis colorectal cancer (HNPCC) is the most common hereditary form of colon cancer, representing 2 to 5 percent of the total. It is caused by alteration of one of the mismatch repair (MMR) genes, a group of genes that function together to proofread DNA as it is copied during replication. In addition to the hereditary setting of HNPCC, some 10 to 15 percent of all nonhereditary colorectal cancers also acquire alterations in these genes. Thus, acquired genetic alterations limited to the colorectal cancer itself and absent from the patient’s germline DNA are far more common than germline HNPCC alterations of the mismatch repair genes. Nonetheless, both germline and acquired alterations result in tumors with the same pathology and biology (1-12).

Genetic Basis of HNPCC and Microsatellite Instability

The mismatch repair proteins form a complex of at least six molecules that function together to proofread replicating DNA. The complex detects and repairs copying errors introduced by the DNA polymerase each time a cell divides.

DNA polymerases possess inherent copying error rates. If the mismatch repair proofreading complex is defective, non-synonymous mutations are introduced into the DNA, leading to genetic instability.

Microsatellite markers are useful in identifying non-synonymous mutations introduced into the DNA. They are superimposed, and two yellow fusion signals are observed. In the presence of a translocation involving the IGH gene, however, the flanking probes become separated and distinct red and green signals are observed. Cases positive for an IGH translocation may then be analyzed by translocation-specific probes to identify the precise translocation present.

For example, a dual-color, dual-fusion probe for t(11;14)(q13;q32) employs one probe labeled in green that hybridizes to the IGH locus, and one labeled in red for the CCND1 gene. Normal cells show two red and two green signals; in the presence of t(11;14)(q13;q32), two yellow fusion signals are seen, corresponding to the derivative chromosomes. A similar probe is available for t(4;14)(p16.3;q32), and probes for other IGH translocations could also be employed.

The combination of FISH and immunofluorescence is a powerful tool for analysis of specific cell populations that assists in the workup of multiple myeloma. In the future, this technique also could be applied to a wide variety of other neoplasms.

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Figure 2. Analysis of multiple myeloma with FISH for t(11;14)(q13;q32) and simultaneous CD138 immunofluorescence. In a normal plasma cell (panel A), two red signals (CCND1 gene) and two green signals (IGH locus) are seen. In the presence of t(11;14)(q13;q32), two yellow fusion signals are seen corresponding to the two derivative chromosomes in addition to normal red (CCND1) and normal green (IGH) signals (panel B).
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(due to a mutation or methylation abnormality in one or more of its component proteins) the DNA polymerase copying errors accumulate within the genome as mistakes and go unchecked. Such copying errors commonly affect microsatellite DNA sequences — short, highly repetitive stretches of DNA that are scattered throughout the genome that normally are stably inherited. Replication copying errors in microsatellites cause them to become longer or shorter and, therefore, unstable.

Detection of these length changes comprises the basis for the microsatellite instability (MSI) test. MSI is the most sensitive HNPCC screening test available, and it detects 95 to 95 percent of cases (1-14). MSI detects changes in the size of microsatellite sequences in test tissue (tumor) relative to corresponding normal control tissue. Patient-matched normal (e.g. blood) and lesional tissue both are required for MSI testing.

Current consensus criteria and primer sets have been established for diagnosing MSI in colorectal tumors, based on five specific microsatellites. MSI tests results are reported based on the number of altered microsatellites in the tumor relative to normal DNA (Figure 1). The three possible results are: a) microsatellite stable (MSS) when all five tested consensus microsatellites match normal (the normal result); b) negative for high microsatellite instability when only one microsatellite of five is unstable; and c) positive for high microsatellite instability (MSI-H) when two or more consensus microsatellite loci are unstable. The negative for microsatellite high category (b above) is controversial regarding its clinical significance, but most evidence indicates that this finding also is essentially normal. The high microsatellite instability (MSI-H) category is abnormal and indicative of either HNPCC or an acquired alteration in one of the mismatch repair proteins that is limited to the tumor.

Figure 2: Immunohistochemical stains for MLH1 (A) and MSH2 (B) in an adenomatous polyp. Note that in both panels, the stromal cells within the lamina propria exhibit internal positive control normal staining, indicating that the stain worked appropriately. Alternatively, the adenomatous epithelium reveals normal nuclear expression of MLH1 (A, black arrowhead), but loss of nuclear staining for MSH2 (B, black arrowhead). This result implicates an MSH2 gene defect in this patient. Further, because it is evident in a precursor adenomatous polyp, these results indicate an MSH2 germline alteration. This result permits mutation screening to be focused onto MSH2 alone and thereby improves the cost-effectiveness of the analysis. (Immunohistochemical stain using a DAB chromagen with nickel chloride enhancement and methyl green counterstain)

Mismatch Repair Gene Immunohistochemistry

Immunohistochemical screening for loss of mismatch repair protein expression is another available screening test for both HNPCC and acquired microsatellite instability in sporadic colorectal cancer. (9,14). It is less sensitive than MSI testing. The lesser sensitivity is because MSI is a functional test of the entire multiple protein mismatch repair complex and immunohistochemistry examines each protein individually.

Only the most commonly affected proteins with reliable diagnostic antibodies are testable by immunohistochemistry. These proteins include, most importantly, MLH1, MSH2, MSH6, and PMS2, which combined account for an estimated 95 percent of the altered genes in HNPCC. The gene alterations, however, do not always equate with altered immunohistochemistry, as mutated proteins with altered function sometimes remain detectable by immunohistochemistry and thereby yield false testing results. Thus, MSI testing is slightly more sensitive still than immunohistochemistry.

The nuclei of all morphologically normal cells are stained intensely immunohistochemically for these proteins, providing the cells have at least one intact copy of the mismatch repair gene (Figures 2a and b). Lesional cells, on the other hand, with two altered mismatch repair gene copies, generally lose immunohistochemical reactivity for the altered nuclear protein (Figure 2b). Adjacent, non-neoplastic cells within the stroma or muscularis provide a highly useful internal positive control for this staining technique (Figures 2a and b). Tumor loss of expression should be interpreted only if the surrounding stromal and/or lamina propria cells reveal appropriate positivity.

Because the immunohistochemical tests are less sensitive than MSI, they are better used to pinpoint the actual defective gene in any given patient with high microsatellite instability, rather than as the first-line screening test. MSI PCR testing and immunohistochemistry for these multiple proteins have comparable costs, dispelling the common misconception.

Figure 3: HNPCC colonic adenocarcinoma with intraepithelial lymphocytes, also known as tumor infiltrating lymphocytes (TIL’s), denoted by the black arrowheads. This feature is highly suggestive of HNPCC.

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that the PCR-based test is much more expensive.

Cost-benefit analysis favors MSI testing first, followed by immunohistochemistry. By pinpointing the culprit gene, immunohistochemistry eliminates the major added expense and effort entailed in finding the actual causative mutation or methylation alteration among the numerous mismatch repair proteins in the complex.

Ultimate identification of the mutation or methylation alteration is the third and final step of testing in this algorithm. Final identification of the actual mutation (or methylation alteration) in a family allows genetic testing of remaining at-risk family members for germline alterations using simple blood or buccal swab DNA samples (14).

**HNPCC Screening Test Requirements**

Both of the two screening phenotypic tests discussed above, MSI and immunohistochemistry, require lesional (adenomatous or carcinomatous) tissue. Formalin-fixed, paraffin-embedded tissues can be tested. Because normal and lesional tissues are required, these screening tests are far more restrictive than germline testing of at-risk family members. Fortunately, germline testing can utilize any normal tissue alone (such as a buccal swab or blood or other paraffin blocks), provided that the causative mutation or methylation abnormality can be identified in the index patient.

Lesional tissue is required for the screening tests because HNPCC patients inherit one abnormal allele in their germline DNA. It appears that their other remaining functional copy is largely sufficient to maintain normal cell function and morphology. Thus, normal tissues in an HNPCC patient all have one altered germline copy but lack MSI under normal conditions and exhibit normal morphology and immunohistochemical nuclear staining of the mismatch repair proteins, based on their one functioning allele.

Tumors appear to develop when the second allele also becomes altered. When both alleles are defective, the affected cells begin to accumulate mutations and form tumors. MSI then becomes detectable within the tumor and there is loss of the affected mismatch repair protein in the lesional cells by immunohistochemistry.

Tumors develop in one or more of the organs at risk for cancer in the HNPCC syndrome. The basis for the observed organ specificity in HNPCC is not well understood at present (10). Nonetheless, the syndromic tumors in HNPCC include colon cancer in 80 to 100 percent of patients; endometrial cancer in an estimated 50 percent of women with HNPCC; and lesser numbers of small intestinal, biliary, urinary bladder, and pancreatic carcinomas, sebaceous skin neoplasms, and glioblastoma multiforme (13,14). Any of these lesional tissues may be used for the HNPCC screening tests of MSI and mismatch repair protein immunohistochemistry (9,13,14).

**Testing of Precursor Lesions**

Each carcinoma in the syndromic tumors of HNPCC develops from a premalignant precursor lesion. In the large and small intestines, the precursor lesion is the adenomatous or dysplastic polyp. The name, hereditary non-polyposis colorectal cancer does not imply there are no polyps, rather that there is no polyposis. Adenomatous polyps are present but are fewer in number.

Adenomas also develop in the stomach and small intestines of HNPCC patients. Isolated adenomas of the stomach and small bowel are quite rare outside the setting of HNPCC and FAP. As such, adenomas at these sites should prompt diagnostic testing. The other fairly common precursor lesion that can also be used for HNPCC screening is endometrial hyperplasia, the precursor of endometrial adenocarcinoma.

Screening tests for hereditary purposes are best done on precursor lesional tissue, if available. Positive results on precursor lesions are more specific than carcinomas for germline mutations, based on the fact that carcinomas also may harbor the three to five times more common acquired mismatch repair gene defects that are not present in germline DNA. The increased specificity of precursor lesions for germline mutations is based upon the observation that acquired mismatch repair gene alterations are a late event in sporadic colorectal cancer and accordingly are very rare in sporadic adenomas (11,12). Virtually all sporadic adenomas, therefore, lack MSI and have intact mismatch repair proteins by immunohistochemistry. This contrasts with adenomas arising in HNPCC, which have MSI in the majority and loss of mismatch repair expression by immunohistochemistry.

HNPCC patients also may develop sporadic-type adenomas unrelated to the Continued on page 6
match repair pathway. Accordingly, the sensitivity for HNPCC adenoma testing, especially for small adenomas, is imperfect and estimated at approximately 66 percent (15). Consequently, normal results from precursor lesion testing are less helpful than abnormal results, which are compelling evidence for a germline alteration (HNPCC). The specificity for germline alteration is improved by testing precursor lesions, even though the sensitivity is somewhat diminished relative to carcinoma testing.

Testing of cancers in HNPCC is also very important, however, because precursor lesions are not always available for testing, and sporadic-type adenomas can occur in HNPCC patients. The culprit gene in an HNPCC cancer will still guide mutation testing in family members, just as readily as one detected from a precursor lesion. However, it is important to keep in mind that alterations identified in a cancer are at least three to five times more likely than those in an adenoma to represent sporadic, acquired mutations that have no bearing on the germline or remaining family members.

Finally, testing of cancers is important because MSI analysis has implications beyond the hereditary question. Specifically, MSI has been shown to predict stage-independent improved prognosis (8,16), as well as potentially diminished response rates to standard 5-FU-chemotherapy (16). These prognostic and therapeutic considerations are true of both hereditary and acquired types of microsatellite unstable colorectal cancers.

MSI testing addresses three questions: 1) the hereditary question of whether a patient and his or her family are affected by HNPCC, in which case precursor lesions should be tested if available for improved diagnostic specificity of germline involvement; 2) prognosis; and 3) therapeutic issues, in which case cancerous tissue should be tested. For these combined reasons, testing of both precursor lesion, if available, and carcinoma testing is generally advisable.

**Pathology of Microsatellite Unstable Colorectal Carcinoma**

Several morphologic features have been identified that are highly associated with HNPCC tumors and sporadic tumors with high microsatellite instability (MSI-H) (1-8). These include intraepithelial lymphocytes (> 2 per high power field or 400X), also known as tumor infiltrating lymphocytes (TILs) (1-7); a Crohn’s-like lymphoid reaction (1-3); any degree of mucinous change (1-3, 8); good or poor differentiation (1-3); right-sided colonic location (1-3); and absence of dirty necrosis (1). (See Figures 3-5 and Table 1.) These morphologic features, along with patient age less than 50 years; right-sided colorectal cancer location; family history of colorectal cancer and/or other tumors within the HNPCC syndrome, including endometrial, small intestinal, biliary, urinary bladder, pancreatic, sebaceous skin neoplasms, and glioblastoma multiforme should prompt MSI testing. MSI testing is performed at The Cleveland Clinic on all colorectal cancers or gastrointestinal adenomas with any of the above pathologic or clinical features suggestive of HNPCC.

**REFERENCES:**


**Table 1. MULTIVARIATE ANALYSIS OF HISTOLOGIC FEATURES IN MSI-H COLORECTAL ADENOCARCINOMAS**

<table>
<thead>
<tr>
<th>Pathologic Feature</th>
<th>p value</th>
<th>Odds ratio</th>
<th>95% confidence limit</th>
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<tr>
<td>&gt; 2 TIL*/HPF**</td>
<td>&lt;0.0001</td>
<td>16.26</td>
<td>5.17, 51.16</td>
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<tr>
<td>Absence of dirty necrosis</td>
<td>0.0054</td>
<td>4.94</td>
<td>1.60, 15.22</td>
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<tr>
<td>Crohn’s-like lymphoid response</td>
<td>0.0064</td>
<td>3.53</td>
<td>1.43, 8.75</td>
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<tr>
<td>Right-sided location</td>
<td>0.0321</td>
<td>2.91</td>
<td>1.09, 7.72</td>
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<tr>
<td>Well or poor differentiation</td>
<td>0.0369</td>
<td>2.70</td>
<td>1.06, 6.85</td>
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<tr>
<td>Any mucinous differentiation</td>
<td>0.0393</td>
<td>2.69</td>
<td>1.05, 6.89</td>
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*Tumor infiltrating lymphocytes; ** High-power field (400X)

Prostate cancer (PCa) is the most common non-cutaneous malignancy and the second leading cause of cancer death among American men. Despite an initial response to androgen deprivation, the advanced stage, metastatic PCa progresses and become hormone-refractory disease.

Historically, chemotherapy for PCa has been reserved primarily for palliation of symptoms. This approach is changing, however, with several novel pharmacological strategies and different chemotherapy regimens under investigation to improve survival and response in PCa patients. These include cytotoxic agents; vaccine therapy; prostate-specific membrane antibodies; bisphosphonates; and biological or targeted therapies, including microtubule inhibitors alone or in combination with other experimental agents such as calcitriol, thalidomide, or flavopiridol.

Investigative Therapies

Docetaxel (Taxotere, Aventis Pharmaceuticals, Bridgewater, N.J.), a member of the taxane family, is a microtubule depolymerization inhibitor. By altering the equilibrium between microtubule assembly and disassembly during mitosis, Taxotere impairs mitosis and cell proliferation in the tumor and induces apoptosis. Recent studies have found that the combination of docetaxel and estramustine regulates the expression of genes important for cell growth, apoptosis, differentiation, oncogenesis, invasion, and metastasis.

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a cytokine that regulates the proliferation and differentiation of myeloid precursor cells. GM-CSF can influence the recruitment, activation, and survival of macrophages and dendritic cells. Experimental data suggest that GM-CSF may increase the efficiency of tumor antigen presentation and the subsequent activation of tumor-specific, cytotoxic T-lymphocytes. Thalidomide is a sedative and an anti-inflammatory agent with antiangiogenic activity. The potential utility of combining GM-CSF and thalidomide in the treatment of advanced PCa has been recently assessed in a phase II clinical trial with promising results.

PTEN’s Potential

Initiation and progression in prostate carcinogenesis depends predominantly on the inability of prostatic epithelial cells to undergo apoptosis. Reversal of the molecular defect in this process, therefore, could prove to be of great therapeutic importance for PCa patients.

The tumor suppressor gene PTEN, located on chromosome 10, encodes a lipid phosphatase that negatively regulates cell growth and/or proliferation by negatively regulating the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway. Loss of PTEN protein expression occurs frequently in PCa. It may be associated with chemotherapy resistance and also serve as a useful independent prognostic marker.

Using high-throughput tissue microarrays, we examined the expression of PTEN by immunohistochemistry in 157 moderately to poorly differentiated (Gleason score 7, GS7) PCa cases; 49 PCa cases with concomitant or subsequent metastatic PCa (Met); and 44 clinically advanced PCa cases treated with neoadjuvant therapy, including 17 patients who received a combination of GM-CSF and thalidomide (GM-CSF/thal), and 27 who received Taxotere (Tax). Granular cytoplasmic staining for PTEN was detected in the secretory cells of benign prostatic glands. Based on the pattern of staining, the PCa cases were initially divided into three groups: positive (the entire tumor showed staining); mixed (both positive and negative tumor cells/glands were present); and negative (no staining was seen in the represented tumor) (Figure 1).

PTEN protein expression was lost
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(negative) in 47.8 percent of GS7 and 44.9 percent of Met, and its expression was reduced (mixed) in 42.7 percent of GS7 and 51 percent of Met PCa. The difference between GS7 and Met PCa was not significant.

PTEN and Neoadjuvant Therapy
Interestingly, PTEN expression was altered by neoadjuvant therapy. Compared to GS7 and Met, PCa cases treated with taxotere and GM-CSF had significantly increased PTEN expression (Tables 1 and 2). Only 23.5 percent of GM-CSF/thal and 18.5 percent of Tax PCa had negative PTEN staining.

The cases subsequently were divided into those showing positive staining (positive and mixed groups) and those with total absence of staining (negative group), and the results of PTEN expression in each group were compared (Table 3). PTEN expression was significantly higher with Taxotere treatment (GS7 vs Tax, p=0.0040; Met vs Tax, p=0.0213).

Conclusions
Our data suggest that the vast majority (90 percent of GS7 and 96 percent of Met) of untreated PCa cases had lost PTEN expression in at least a portion of the tumor cells. This is in keeping with the marked heterogeneity of PTEN expression reported in the literature. We also found PTEN expression significantly increased by neoadjuvant treatment, particularly Taxotere, suggesting that the drug affects the PTEN pathway and, therefore, may have therapeutic implications.

The mechanism by which Taxotere increases PTEN expression in PCa is unclear. Taxotere may kill PTEN negative cells preferentially, those with more aggressive biological behavior that are more likely to become metastatic clones. Alternatively, PTEN expression may be restored by Taxotere treatment. Because PTEN may be inactivated by epigenetic mechanisms other than gene deletion and mutations, it is conceivable that inactivation of PTEN by epigenetic mechanisms could be reversed by Taxotere treatment.

In view of PTEN’s function as a regulator of apoptosis or cell survival and an inhibitor of cell cycle progression, coupled with our finding that its expression is significantly altered by neoadjuvant therapy, PTEN functional status may have important therapeutic and prognostic implications for PCa.

Table 1: Expression of PTEN in different PCa groups

<table>
<thead>
<tr>
<th>PTEN</th>
<th>GS7</th>
<th>Met</th>
<th>GM-CSF/thal</th>
<th>Tax</th>
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<td>Positive</td>
<td>15</td>
<td>2</td>
<td>4</td>
<td>1</td>
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<tr>
<td>Mixed</td>
<td>67</td>
<td>25</td>
<td>9</td>
<td>21</td>
</tr>
<tr>
<td>Negative</td>
<td>75</td>
<td>46</td>
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Table 2: Comparison of PTEN Expression (Positive, Mixed, and Negative) in different PCa groups

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<th>Variable 2</th>
<th>p-value</th>
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<td>GS7</td>
<td>Met</td>
<td>0.41</td>
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<td>GM-CSF</td>
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<td>Tax</td>
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<tr>
<td>Met</td>
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<td>Fisher’s</td>
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Table 3: Comparison of PTEN Expression (Positive + Mixed vs Negative) in different PCa groups

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<th>Variable 1</th>
<th>Variable 2</th>
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<td>GS7</td>
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<td>GM-CSF</td>
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<td>Tax</td>
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<td>Tax</td>
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