Loss of Fragile Histidine Triad Gene Expression in Advanced Lung Cancer Is Consequent to Allelic Loss at 3p14 Locus and Promoter Methylation

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Abstract
The fragile histidine triad (FHIT) gene located at the 3p14.2 locus plays an important role in the pathogenesis of lung cancer. The objective of this study was to analyze loss of heterozygosity and FHIT gene methylation status and correlate them to fhit expression. Bronchoscopically obtained lung biopsies from 30 cases of histologically proven carcinoma of the lung in stage III were assessed for the alterations in the FHIT gene. Fhit protein expression was determined by immunohistochemistry, and transcript levels were determined by reverse transcription-PCR. Microsatellite alterations and methylation status of the Fhit gene promoter were determined by PCR. Loss of heterozygosity at the 3p14 locus was observed in all the 30 cases at least by one of the three microsatellite polymorphic markers. The FHIT gene promoter showed complete methylation in 37% cases and partial methylation in 47% cases, and 16% cases showed no promoter methylation. FHIT full-length coding region (exons 5-9) transcripts were present in eight cases (26.6%), and aberrant transcripts were additionally seen in four cases. Loss of FHIT mRNA expression correlated to FHIT promoter methylation but not to loss of heterozygosity at the 3p14 locus. There was a strong correlation between the expression of FHIT at the transcript and protein level. The apoptotic index estimated by the terminal deoxynucleotidyl transferase–mediated nick end labeling assay was significantly correlated to the fhit protein expression. The results of this study indicate that in locally advanced carcinoma of the lung, there is frequent loss of FHIT expression, and methylation of the FHIT gene promoter is an important mechanism of its inactivation. (Mol Cancer Res 2006;4(2):93–9)

Introduction
Lung carcinoma is an important cause of cancer-related deaths worldwide with a rising incidence in the developing countries as well (1). Tobacco smoking is the major etiologic factor for lung cancer (2). The development of human cancer is a multistep process involving the clonal evolution of abnormal cell populations that gain a selective advantage over normal cells by accumulating specific alterations in at least two groups of genes, the proto-oncogenes and the tumor suppressor genes (3, 4). It is estimated that about 10 to 20 genetic events are required for lung tumorigenesis (5). Numerous genetic alterations have been identified that occur at high frequencies in non–small cell lung carcinoma (NSCLC) and SCLC. These include p53 gene mutations (6, 7), k-ras proto-oncogene mutations (8), and alterations in p16 (9), CDC25 (10), and FHIT (11). FHIT, the tumor suppressor gene at the 3p14.2 locus (12) encompasses the FRA3B fragile site in the third intron and is a common target of deletions in primary human cancers, including lung cancer (13). The FHIT gene is composed of 10 exons that span ~2 Mb, out of which the coding region begins in exon 5 and ends in exon 9. It encodes a small mRNA (1.1 kb) and a small 147 amino acid protein with a mass of 16.8 kDa.

Cytogenetic analysis has established that genetic alterations on the short arm of chromosome 3 are among the most common abnormalities found in lung cancer. Loss of heterozygosity (LOH) analyses using polymorphic microsatellite DNA markers are frequently used to identify allelic losses at specific chromosomal regions. Allelic losses at chromosomal region 3p occur relatively early during the multistage development of invasive lung cancer (14). It has also been observed in NSCLC that certain fraction of microsatellites shows either an expansion or a constriction in one or both alleles of the FHIT gene (15).

However, specific correlation to the expression of the fhit protein has not been shown. The expression of FHIT mRNA is detectable in most normal human tissues, and the highest levels of expression are detectable in epithelial cells and tissues (12, 16). In tumor-derived cell lines and tumor tissues, genomic deletions often have resulted in the absence of exons in the FHIT mRNA (16). Aberrant methylation of normally unmethylated CpG islands, located in the 5′ promoter regions of genes, has been associated with transcriptional inactivation of several genes in human cancer and can serve as an alternative to mutational inactivation (17). Hypermethylation of the FHIT gene promoter leading to its transcriptional silencing has been previously reported (16). In lung cancer, loss of the fhit protein...
Table 1. Analysis of LOH Using Microsatellite Polymorphic Markers to the Chromosome 3p14 Locus

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NOTE: +, LOH present; -, LOH absent.
Abbreviations: UI, uninterpretable; Tm, tumor sample; BAL, bronchoalveolar lavage fluid.

Results

A total of 30 lung cancer biopsies and the corresponding bronchoalveolar lavage fluid were analyzed for FHIT alterations. The patients included in the study were all males and smokers with median age of 56.08 years (range, 40-80 years). There were 27 cases histologically typed as squamous cell carcinoma and three cases as SCLC. Cytologic examination of the bronchoalveolar lavage fluid showed malignant cells in four cases and was negative in the other cases. Genomic DNA derived from the tumor tissue bronchoalveolar lavage fluid sediment was analyzed for microsatellite alterations and compared with the control DNA derived from the peripheral blood lymphocytes as described in Materials and Methods.

Microsatellite Alterations Using Polymorphic Markers

The microsatellite polymorphic markers D3S1300, D3S1312, and D3S1313 were analyzed by PCR for microsatellite alterations. The results are summarized in Table 1, and representative gels are depicted in Fig. 1. D3S1300 is an intragenic marker located in intron 5, whereas D3S1312 is flanking exon 1, and D3S1313 is located near exon 10. LOH was observed at least by one of the abovementioned polymorphic markers in all the 30 cases studied. Two, three, and one tumor case was noninformative using the D3S1300, D3S1312, and D3S1313 markers, respectively. Using the D3S1300 marker, the frequency of LOH was 40% in biopsies versus 6.6% in the bronchoalveolar lavage fluid. Using the D3S1312 marker, it was 63% in tumors and 20% in the bronchoalveolar lavage fluid, whereas at the D3S1313 locus, LOH was present in 73% biopsies and 36% bronchoalveolar lavage fluid samples. Microsatellite instability was observed in a fewer number of cases compared with LOH. Using D3S1300 marker, microsatellite instability was noted in four tumor cases, and in one case, this was seen in the corresponding bronchoalveolar lavage fluid as well. With D3S1312 marker, microsatellite instability was seen in three tumor cases and in one case in the matching bronchoalveolar lavage fluid. With the same marker, one bronchoalveolar lavage fluid case showed microsatellite instability, which was not present in the tumor. Using the D3S1313 marker, there was one case with microsatellite instability in both the tumor and bronchoalveolar lavage fluid, one case with microsatellite instability present only in the tumor, and three cases where microsatellite instability was seen in the bronchoalveolar lavage fluid only. There was no correlation of LOH detected by any of the three microsatellite polymorphic markers individually to the FHIT transcript and protein expression.

FHIT Gene Promoter Methylation

Using previously published primers (18), we determined the frequency of FHIT gene promoter methylation in tumor biopsies of lung cancer patients, and the results are shown in Fig. 2 and are tabulated in Table 2. Out of the 30 cases studied, 11 cases showed complete gene promoter methylation (37%), whereas in 14 cases, there was partial methylation (47%). In five cases, (16%), the FHIT gene promoter was unmethylated. The methylation of the FHIT gene promoter correlated significantly to absence of the FHIT transcript as determined by reverse transcription-PCR (Pearson’s correlation, \( P = 0.004 \)). Conversely, the FHIT transcript was present only if the gene promoter was unmethylated or partially methylated.

FHIT mRNA Expression

FHIT mRNA was amplified as previously described (12). The coding exons are represented by exons 5 to 9, and this region was amplified by reverse transcription-PCR. Very low levels to undetectable levels were observed in majority of the cases (Fig. 3). In all cases, the cDNA corresponding to the \( \beta \)-actin housekeeping gene was observed to be amplified. PCR was repeated thrice to confirm the results along with a positive control sample. Thus, out of 30 cases of lung cancer analyzed, eight cases showed the amplification of full-length coding region transcript of FHIT mRNA, and the remaining 22 cases did not show any product at all (Table 2). Out of these eight cases, aberrant transcripts were seen in four cases in addition to the full-length coding region transcripts.
Fhit Protein Expression

Using immunohistochemistry, we analyzed fhit protein expression in patients with lung cancer. The samples of normal bronchial epithelium and bronchiolar epithelium showed strong cytoplasmic staining for the fhit protein in all the cases and, when present, served as a positive internal control for immunostaining (Fig. 4A). In contrast, the tumor epithelial cells showed negative or reduced fhit protein staining (Fig. 4B and C). Overall, 66% of the tumor biopsies showed no expression of fhit, whereas only 33.3% of the tumor biopsies showed faint or reduced staining compared with normal bronchial tissue (Table 2). In two cases, the fhit protein was detectable as a weak positivity in the tissue sections, although the transcript was not detectable. Overall, however, there was a significant correlation between the FHit transcript and fhit protein expression as determined immunohistochemically (Pearson’s correlation, $P < 0.001$).

Terminal Deoxynucleotidyl Transferase–Mediated Nick End Labeling Assay for Apoptosis

The terminal deoxynucleotidyl transferase–mediated nick end labeling assay was carried out to detect apoptotic nuclei in situ in the tissue sections. There were variable number of apoptotic cells in the tumor studied (Fig. 4D). The apoptotic index (number of apoptotic cells per 100 tumor cells) ranged from <0.1 to 2.6, with a median of 0.5. The apoptotic index correlated significantly with the immunohistochemical expression of the fhit protein (Pearson’s correlation, $P = 0.039$).

Discussion

This study is a detailed analysis correlating the expression of the FHit gene at the transcript and protein levels to the LOH at the 3p14.2 locus and FHit gene promoter methylation in cases of lung cancer.

Frequent alterations of FHit expression occurs in the form of exon deletions involving one or both alleles of the FHit gene in a variety of human cancers. Previous studies have indicated that >90% of the lung tumors show LOH at the FHit locus, which is confirmed by this study. The D3S1313 marker was the most frequently altered marker followed by D3S1312 and D3S1300 in the primary tumor as well as the corresponding bronchoalveolar lavage fluid. However, two of the three markers (i.e., D3S1312 and D3S1313) were positive in 48% cases, whereas 100% of the lung tumor biopsies studied showed LOH for at least one of the three markers studied. The proportion of bronchoalveolar lavage fluid showing LOH was 6.6% with D3S1300, 27% with D3S1312, and 36% with D3S1313. Hence, LOH studies on cell sediments of the bronchoalveolar lavage fluid can supplement conventional cytologic techniques for detection of malignancy. In a previous study, it has been shown that the sensitivity of cytologic detection can be improved by LOH analysis using D3S1300 marker (19). In two cases, LOH was detected in the bronchoalveolar lavage fluid samples but not in the tumor biopsies. Such a finding has also been reported by Ahrendt et al. (20) in up to 17% cases. Tumor heterogeneity could be a possible mechanism for such a discrepancy. LOH at the FHit locus is reported higher in smokers with lung cancer compared with nonsmoker (21). Such an analysis was not possible in this study as all the patients were chronic smokers with high smoking indices.

After observing the allelic imbalance in the FHit gene at genomic level, we next assessed the alterations at the transcript and protein level. In the present study, using a non-nested single-step PCR approach to amplify only the coding exons of FHit, we observed FHit full-length coding transcripts in 8 of 30 cases (27%). In four cases, there were aberrant smaller-sized transcripts in addition to the normal transcript. In NSCLC, presence of both aberrant and normal FHit transcripts were seen in 40% (11), 59% (22), and 100% tumors (23). Sequence analysis of aberrant products showed that majority of these products lacked either exon 5, which contains the start codon, or exon 8, containing the histidine triad motif important for its functional activity. In 73% of our cases, no FHit transcript could be amplified. This could be because of differences in methodology (i.e., nested versus non-nested PCR approach). Some authors believe that nested PCR results in greater frequency of

![FIGURE 1. Analysis of microsatellite alterations (LOH/microsatellite instability) in paired normal (N), in bronchoalveolar lavage fluid (B), and in tumor (T) biopsies using the polymorphic markers. A, D3S1300. B, D3S1312. C, D3S1313. A and F. Additional bands (arrow) are present in the tumor lane that are not present in the corresponding normal lane, indicating microsatellite instability. No band was amplified in the bronchoalveolar lavage lane in (A); hence, this lane is not interpretable. F. The bronchoalveolar lavage lane also shows faint additional bands. B, C, and D. The upper or the lower alleles (arrows) are absent in the tumor lane, indicating LOH. The bronchoalveolar lavage lane in (C) shows LOH with respect to the upper allele. E. There is only an apparent reduction in the staining intensity, which is consistent with LOH. Bottom, the gel represents the β-actin gene, which shows that equal amount of DNA has been used for every PCR reaction.](https://www.mcr.aacrjournals.org/content/4/2/95.full.pdf)

detection of aberrant transcripts (24) compared with non-nested PCR. Furthermore, we have chosen to amplify only the coding exons (exons 5-9) of the Fhit cDNA. Hence, if the aberrant transcripts lack exon 5, our primers would be unable to anneal and would result in nonamplification of any product. The second common type of aberrant products are those lacking exon 8 followed by variable skipping of exons 3 to 9 (22). Such aberrant products were observed in four cases in this series. Aberrant transcripts have also been described in histologically normal lung tissue adjacent to primary lung cancer and in metastatic lung cancer; thus, some authors believe that they are not specific to primary lung cancer (22). However, some believe that the presence of aberrant transcript reflect genomic level abnormality in at least one allele (16). Normal Fhit transcript may also be derived from the contaminating stromal and inflammatory cells, but in our studies, all biopsies subjected to molecular analysis showed >90% tumor, and we believe that the transcripts are derived from the tumor cells only, as there was a good correlation between Fhit transcripts and fhit protein expression ($P < 0.001$).

Lack of Fhit gene expression and consequent protein expression can be due to deletions, mutations, or epigenetic modifications, such as methylation. Mutations are not observed frequently in the Fhit gene in primary lung cancers (25). However, loss of protein expression has been reported in 34% to 73% in NSCLC, 73% in SCLC, and 100% in carcinoma in situ (13, 26). Thus, we explored methylation as a mechanism of loss of gene expression. The Fhit gene was fully methylated in 37% and partially methylated in 47% of the cases and showed a high correlation with loss of Fhit expression only at the transcript level ($P = 0.004$). The reported frequency of Fhit methylation ranges from 31% to 45% in the case of NSCLC (18, 27-29) and has been correlated to loss of its expression, which is corroborated by our study. In one report, it has been documented that differential patterns of Fhit methylation were observed in neoplastic versus adjacent nonneoplastic tissues, which points that targeted MSP amplification could be useful in the treatment or prevention protocols (30). Thus, we believe that methylation of the Fhit gene promoter may be an important mechanism of loss of gene expression in carcinoma of the lung. In fact, Maruyama et al. (27) believe that the hypermethylation of the Fhit gene can be used as a prognostic marker in NSCLC.

The relationship of apoptotic pathways to Fhit is not very clear, although previously, the activation of caspase-8 was correlated with Fhit alteration–mediated apoptosis. This suggested that Fhit might exert a proapoptotic function through a caspase-mediated pathway (31). Thus, we evaluated apoptotic index in all cases in this study, which ranged from 0.1 to 2.6. This is consistent with previous reports of apoptotic index in NSCLC (32, 33). There was a positive correlation of the apoptotic index with fhit protein expression, indicating a possible proapoptotic function for the fhit protein (34). The fhit protein is believed to function as an AP3A hydrolase, a molecule that might be involved in the regulation of DNA replication and cell cycle (35). It has been suggested that in tumors where the fhit protein is absent, AP3A cannot be hydrolyzed, which results in elevated levels of AP3A; therefore, it may contribute to carcinogenesis. Hence, our findings that suppression of apoptosis in tumors lacking the fhit protein may further contribute to tumorigenesis warrant further investigation at the cellular level to discern the relationship of Fhit to apoptosis.

Materials and Methods

A total of 30 cases of lung cancer were studied in detail for Fhit alterations. In addition, the bronchoalveolar lavage fluid from these patients was analyzed for LOH at the Fhit locus as described below. These patients were recruited between January
and December 2002 at the Department of Pulmonary Medicine of Post Graduate Institute of Medical and Educational Research (Chandigarh, India). This study was carried out after its formal clearance by the institute’s medical ethical committee. All patients underwent thorough examination and computed tomography scan for staging the tumor. Routinely, fiber optic bronchoscopy was carried out in all patients, after an informed consent. After visualization of the tumor, multiple (at least three to four) biopsies were taken from each patient. One of these was snap frozen and stored at -80°C for molecular analysis, and the remaining two to three tissue pieces were immersed in buffered formalin and subjected to routine histopathologic evaluation. After confirmation of the histologic diagnosis of lung cancer classified as per the WHO guidelines (36), we proceeded with the molecular evaluation. The bronchoscopically obtained biopsy pieces subjected to molecular analysis were confirmed to be representative of the tumor by one frozen section. Only those tissue pieces showing >90% tumor was included in the analysis. In all the cases, bronchoalveolar lavage was done, and fluid was transported on ice to the laboratory and centrifuged. From the sediment, genomic DNA was extracted as described previously (37).

**LOH Using Microsatellite Markers**

Genomic DNA was extracted from the tumor biopsies, bronchoalveolar lavage fluid, and 2 mL of peripheral blood as described previously (37). In each case, the peripheral blood mononuclear cells served as a positive control, against which the tumor and bronchoalveolar lavage fluid sediments were compared. The microsatellite polymorphic markers D3S1300, D3S1312, and D3S1313 amplify regions in chromosome 3p14 location containing CA nucleotide repeats (12, 38, 39). PCR was carried out using 200 ng of genomic DNA, 2 mmol/L deoxynucleotide triphosphates, 1× PCR buffer, 100 pmol of forward and reverse primers, and 1 unit of Taq polymerase (Roche GmbH, Mannheim, Germany) in a total reaction volume of 50 μL. Samples were subjected to initial denaturation at 94°C for 3 minutes followed by 35 cycles of amplification for 45 seconds at 94°C, 45 seconds at 57°C, and 1 minute at 72°C with a final extension at 72°C for 10 minutes. PCR products were electrophoresed on 6% non-denaturing PAGE and was visualized by silver staining. LOH score was based on the absence of alleles in the DNA derived from tumor biopsies and bronchoalveolar lavage fluid, or a

**FIGURE 3.** Reverse transcription-PCR analysis for FHIT transcript in seven cases. Bottom, 382-bp product for β-actin, a housekeeping gene. Lanes 1-3, a 790-bp full-length FHIT coding region transcript (exons 5-9) is observed along with aberrant smaller transcripts in lanes 1 and 2. Note the complete absence of products in lanes 4-7.

**FIGURE 4.** A-C. Fhit protein expression in lung cancer. A. Normal bronchial epithelium (arrow) showing strong fhit positivity with weak immunoreactivity in the underlying tumor. Original magnification, ×100. B. Tumor negative for fhit expression. There was partial methylation of the gene. C. Tumor positive for fhit (avidin-biotin peroxidase), which showed unmethylated alleles. Original magnification, ×200. D. Terminal deoxynucleotidyl transferase–mediated nick end labeling showing apoptotic nuclei (arrow) in a representative case. Original magnification, ×400.
difference in the relative intensities of alleles in the tumor and bronchoalveolar lavage–derived DNA upon comparison with the DNA derived from the normal peripheral blood mononuclear cells by at least 50%. PCR was repeated twice to confirm the LOH in each case.

**Fhit Gene Methylation Analysis**

DNA extracted from the tumor tissues of the lung was subjected to bisulfite modification as reported by Herman et al. (40). Briefly, 2 μg of genomic DNA was denatured with 0.2 mol/L NaOH and 10 mmol/L hydroquinone (Sigma Chemical Co., St. Louis, MO), and 3 mol/L sodium bisulfite (pH 5; Sigma Chemical) were added and incubated for 16 hours at 50°C. Afterwards, modified DNA was purified using Wizard DNA purification resin (Promega Corp., Madison, WI) followed by ethanol precipitation. Treatment of genomic DNA with sodium bisulfite converts unmethylated cytosines to uracil, which are then converted to thymidine during the subsequent PCR step, producing sequence differences between methylated and unmethylated DNA. Primer sequences were determined based on sequence data of the 5′ CpG island of the gene (Genbank accession nos. U76262 and U76263). The PCR mixtures contained 1× PCR buffer, 1.25 mmol/L deoxynucleotide triphosphates, 100 pmol of each primer, 1 unit FastStart Tag DNA Polymerase (Roche), and 100 ng bisulfite-modified DNA. PCR was done for 40 cycles at 94°C for 1 minute, 68°C for 1 minute, and 72°C for 1 minute, and a final extension for 10 minutes at 72°C was included at the end. DNA from peripheral blood lymphocytes of healthy individuals was used as a positive control for the unmethylated form, and DNA from peripheral blood lymphocytes treated with SsI Methyltransferase (New England Biolabs, Inc., Beverly, MA) was used as a positive control for methylated alleles. A negative control without DNA was included in each set of PCR. The PCR products of 74 bp were separated on 3% agarose gel, stained with ethidium bromide, and visualized under UV illumination. The specificity of the PCR products was confirmed by sequencing some of them (data not shown).

**Reverse Transcription-PCR**

Total RNA was isolated by the parallel DNA/RNA isolation kit (Qiagen, Germantown, MD) from the biopsy tissue. cDNA was synthesized using 1 μg of template RNA, 0.5 μg of oligo dT primer, 15 units avian myeloblastosis virus reverse transcriptase (Roche), 2 mmol/L each deoxynucleotide triphosphates, and 20 units RNase inhibitor. The mixture was incubated at 42°C for 2 hours and subsequently heated for 2 minutes at 94°C. The reaction mixture was then used for PCR. For all samples, a negative control of a similar reaction without avian myeloblastosis virus reverse transcriptase was included. PCR reaction was carried out on 2 μL of the cDNA template using 1× PCR buffer (Roche), 2 mmol/L deoxynucleotide triphosphates, 100 pmol of forward and reverse primers, and 0.75 unit Taq DNA polymerase in a final volume of 25 μL. The primer sequence used were as follows: β-actin sense, 5′-TCTACAATGAGCTGCGT-3′ and antisense, 5′-CTTTAATGCACGCACA-3′ (382-bp product); Fhit (12) sense, 5′-CATCCTGGAGCTTGAAGCTC-3′ and antisense, 5′-TCATGGTTGAAGAATACGG-3′ (790-bp product).

DNA was subjected to initial denaturation at 94°C for 3 minutes. The PCR conditions used were 95°C for 30 seconds, 61°C for 45 seconds, and 72°C for 45 seconds for 35 cycles followed by a final extension at 72°C for 7 minutes. This assay amplified a full-length coding region transcript of 790 bp from exons 5 to 9. The PCR products were separated on 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination.

**Analysis for fhit Protein Expression by Immunohistochemistry**

Immunostaining on formalin-fixed, paraffin-embedded tissue sections was done by the avidin-biotin peroxidase complex method using the avidin-biotin complex staining kit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); 5-μm-thick sections were mounted on poly-l-lysine–coated slides, deparaffinized, and rehydrated through xylene and a graded series of alcohol. The slides were incubated with the blocking solution (0.3% H2O2 + methanol) for 20 minutes to block endogenous peroxidase. Antigen enhancement was done the sections at 100°C in 0.01 mol/L sodium citrate solution (pH 6) for 2 minutes. The slides were cooled under tap water and washed thrice in 0.1 mol/L PBS (pH 7.2) and then incubated with blocking serum for 30 minutes at room temperature. The slides were incubated with primary rabbit anti-Fhit antibody (Zymed Laboratories, South San Francisco, CA; dilution, 1:80) for 2 hours at room temperature. The slides were washed with PBS and incubated with avidin-biotin complex reagent for 10 minutes at room temperature. The reaction products were again washed in PBS and then developed with diaminobenzidine tetrachloride (Sigma Chemical) as a chromogen. The sections were lightly counterstained with hematoxylin followed by dehydration, clearing, and mounting with DPX. The brown reaction product was scored as positive and negative by light microscopy.

**Determination of Apoptosis by the Terminal Deoxynucleotidyl Transferase–Mediated Nick End Labeling Method**

The apoptotic cells were detected by the in situ Cell Death Detection kit (Roche) as per manufacturer’s instructions on 5-μm sections cut from the formalin-fixed, paraffin-embedded tissues. Briefly, cells were fixed and permeabilized with the terminal deoxynucleotidyl transferase–mediated nick end labeling reaction mixture containing the enzyme terminal deoxynucleotidyl transferase and fluorescein-labeled dUTP at free 3′-OH group in single-double and double-stranded DNA. After washing, the label incorporated at the damaged sites of DNA was marked by the anti-fluorescein antibody conjugated with enzyme peroxidase. After removal of unbound enzyme conjugate, the peroxidase retained in the complex was visualized with a precipitating substrate, diaminobenzidine (Sigma Chemical), which gives a dark brown precipitate. The slides were examined under light microscope. Counting of
apoptotic nuclei was done by the Leica Image Analyser using the Quantimet 600 software (Cambridge, England). A minimum of four fields were examined in each case, and the number of apoptotic cells per 100 tumor cells was determined and % apoptotic index was calculated.

References
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