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CLINICAL STUDY

Un-methylation of the survivin gene has no effect on immunohistochemical expression of survivin protein in lung cancer patients with squamous cell carcinoma

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ABSTRACT

Survivin is a member of the inhibitor of apoptosis (IAP) family. The function of the survivin protein is to inhibit caspase activation, thereby leading to negative regulation of apoptosis or programmed cell death. This has been shown by the disruption of survivin induction pathways leading to an increased apoptosis and decreased tumour growth. These data suggest that survivin may provide a new target for cancer treatment, which would distinguish transformed cells from normal cells.

In the present study, we aimed to investigate exon 1 of the survivin gene by means of methylation-specific PCR and evaluate its impact on survivin protein expression following DNA isolation and bisulphite modification in paraffin-embedded normal and tumour tissues of lung cancer patients with squamous cell carcinoma.

We used 41 squamous cancer tissues with methylation in exon 1 of the survivin gene and non-methylation in corresponding tumours. However, the immunohistochemistry staining of these samples demonstrated an increased survivin protein compared to normal tissue. While there is almost no other study to date on this subject matter, we believe that the absence of methylation in exon 1 of the survivin gene may not affect disease prognosis as it has no effect on expression, and possible promoter methylation or transcription factors *(Tab. 1, Fig. 4, Ref. 15)*. Text in PDF *www.elis.sk.*

KEY WORDS: survivin, methylation, lung cancer, methylation, survivin gene, squamous cell carcinoma.

Introduction

Lung cancer is responsible for approximately 20 % of the cancers in USA and is the leading cause of cancer-related mortality across the globe. Ninety percent of lung cancers are non-small cell lung cancers, 30 % of which are squamous cell carcinomas (1).

The human survivin gene is located on the telomeric position of chromosome 17 with a size of 14.7 kb. The survivin gene consists of 4 exons and 3 introns, and encodes the survivin protein of 16.5 kD. While other nhibitors of apoptosis proteins (IAPs) contain more than one BIR (baculovirus IAP repeat domain) in their structure, the survivin gene contains only one BIR region at the N-terminal domain, and it also contains alpha-helix structure at the C-terminal domain. The survivin protein interacts with caspase 3 and caspase 7 via the BIR region, while its alpha-helix structure interacts with tubulin subunits during mitosis (2-3).

DNA methylation is the most widely studied and the most well-established epigenetic mechanism. It is an enzymatic change where cytosines are converted to 5'-methylcytosine. The cytosine-end methylation seen in the mammalian genome often occurs at the 5'-CG-3' dinucleotides, which are also called CpG dinucleotides (4).

In the present study, we aimed to investigate potential methylation changes in exon 1 of the survivin gene in squamous cell tumours compared to normal cells by means of methylation-specific PCR, and to determine its relationship with survivin expression by means of immunohistochemistry methods.

Materials and methods

Cervical tissues were collected from the patients at the Pathology Department of Trakya University Hospital, Trakya University Faculty of Medicine, Turkey. This study has been approved by the university's ethics committee. Study related information was provided and informed consent was obtained from each patient. All cases were histologically examined and confirmed by pathologists. Squamous cell lung tumour staging was performed according to the staging system of International Federation of Lung Cancer Committee.

There were 41 cases of squamous cell carcinoma (SCC), for which the age, histological type, grading, staging, and size of lesion are presented in Table 1.

DNA isolation from paraffin-embedded tissues

Sections of 4 – 10 µm thickness were obtained from formalin-fixed, paraffin-embedded tumours and normal tissues. These sec-

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Tab. 1. Clinical characteristics of the patients.

Patient information	Normal cases (n=85)	Tumour cases (n=85)
Age (Mean±SD)	55±11	
≤53	22 (54%)	22 (54%)
> 59	19 (41%)	19 (41%)
Gender		
Female	7 (17%)	17 (17%)
Male	36 (83%)	36 (83%)
Grade		
1		23 (56%)
2		3 (7%)
3		15 (37%)

tions were transferred to tubes for DNA isolation and xylene was used to remove the paraffin prior to the DNA isolation process. DNA isolation was performed subsequently. Invitrogen DNA isolation kit was used to isolate DNAs.

The DNAs obtained from this procedure were analysed at 280 nm and 260 nm wavelength via spectrophotometric method with NanoDrop; DNA quantities were determined; and the samples were stored at +4 °C to be analysed later.

Bisulphite modification was performed for all isolated DNAs as per Sigma-Aldrich – Imprint DNA Modification Kit protocol.

Methylation analysis of the survivin gene

This method is based on DNA changes triggered by sodium bisulphite. After genomic DNA was obtained, DNA samples were treated with bisulphite for methylation analysis of the survivin gene. By doing so, all unmethylated cytosines were transformed to uracil. Because this modification converts cytosines to thymine in the unmethylated region, MethPrimer V1.1 beta (available on www.urogene.org) program was used for the region thought to be altered this way in order to determine the CpG sequences in exon 1 of survivin and the methylation-specific primer sequences. MSP was performed by means of the following protocols to investigate methylation (Fig. 1) (5).

MSP (methylation-specific PCR) protocols

Methylation-specific primers for exon 1 of survivin gene region: Srv1eMt F: GTTTTTATTTTTAGAAGGTCGCG, Srv1eMt R: CAAACAAAAAACAACGTCGAA, Product Size: 159 bp, Tm: 77.2; unmethylation-specific primers for exon 1 of survivin gene region: Srv1eUnMt F: TTTTATTTTTAGAAGGTTGTGG, Srv1eUnMt R: AAACAAAAAACAACATCAAA, Product Size: 156 bp, Tm: 72.3; PCR condition: Initiation, 95 °C 10' followed by 95 °C 45'', 55 °C 30'', 72 °C 30'' 35 cycles, final 72 °C 5'. Methylated and unmethylated PCR media: PCR buffer 1x, $MgCl_2$: 2 mM, DMSO: %5 (v/v), dNTP: 12.5 mM, Primer Forward: 10 nM, Primer Reverse: 10 nM, Taq Polymerase: 1 U (5 U/μL). Template DNA 100 ng filled up to 50 μL with dH_2O. Methylated and unmethylated human DNAs were used as positive and negative control DNAs (S8001 | CpGenome™ Human Methylated & un-Methylated DNA Standard Set). These DNAs were subjected to modification prior to PCR.

PCR was performed with these PCR samples using primers designed as methylation-specific and unmethylation-specific. The PCR products were assessed under ultraviolet (UV) light on 2 % agarose gel, tumours and normal tissues were compared for methylation, and methylation rates were determined.

Immunohistochemical detection

We used an immunohistochemical method to detect the survivin gene protein. For this purpose, we obtained samples of 5 μL (± 2 μL) on average to polylysine slides from 85 cases. Using Ventana auto-analyser, all sections were treated with survivin protein antibody with Roche and Novus antibody detection kits. Glia astrocytes were used as positive control as recommended in the manufacturer's company data sheet. Internal control was used as negative control and unstained interstitial regions on case slides were accepted as negative. During the evaluation, the most dense areas on case slides were assessed semi-quantitatively and survivin presence was determined in % (percent). According to this

Fig. 1. Upper row – original sequence, lower row – bisulphite-modified sequence (for display, assuming all CpG sites are methylated) ++ CpG sites, :::: Non-CpG 'C' converted to 'T', M >>>>>> Left methylated-specific primer, M <<<<<< Right methylated-specific primer, U >>>>>> Left unmethylated-specific primer, U <<<<<< Right unmethylated-specific primer.

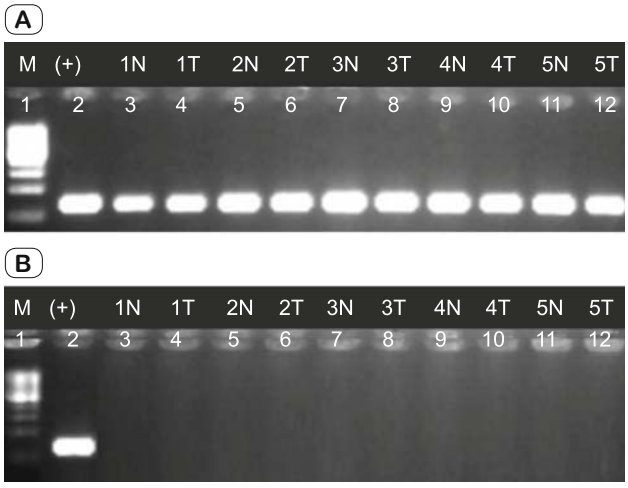

Fig. 2. A) N – normal, T – tumour, unmethylation-specific PCR result of exon 1 of survivin, Line 1 – M: 100 bp marker, Line 2 – (+) Control DNA, normal and tumour pairs of the same cases in Lines 3–12, full non-methylation-PCR signal in N sample and T sample of cases in Lines 3–12. B) Methylation-specific PCR result of Survivin promoter, Line 1 – M: 100 bp marker, Line 2 – (+) Control DNA, methylation (-) in N sample, (-) in T sample of cases in Lines 3–12.

approach, interpretation was as follows: 0–5 % negative (–), 6–25 % (+), 26–50 % (++), 51–75 % (+++), 76–100 % (++++) (Fig. 2).

Statistics

Fisher's exact test and odds ratio (OR) test were used, and P <0.01 was considered statistically significant (6).

Results

1–3 µL bisulphite-modified DNA was used per each methylated and unmethylated PCR reaction. MSP and un-MSP products were assessed on 2 % agarose gel stained with ethidium bromide (Figs 2–4). The expected band size was 159 bp for MSP and 156 bp for un-MSP in exon 1 of the survivin gene.

Furthermore, immunohistochemical staining and analyses were performed with sections on slides from the samples used for PCR. Increased survivin protein expression was detected, showing non-correlation with non-methylation of exon 1 of the survivin gene (Figs 3–4).

Clinicopathological information of 41 patients is presented in the Table 1.

Expression of survivin in squamous carcinoma

While strong-heterogenic nuclear and few delicate shades of cytoplasmic survivin expression were observed in squamous tumour tissues in the lung (Fig. 3), weak-nuclear and cytoplasmic survivin expression was seen in alveolar epithelium (Fig. 4). However, methylation in exon 1 of the survivin gene was not observed for both bronchial and squamous tumour tissues of the same cases (Fig. 2 A,B).

Survivin-positive nuclear staining was observed in 83 % of squamous carcinomas, and cytoplasmic survivin expression was detected in 33 %. Expression of nuclear and cytoplasmic survivin was significantly higher in squamous carcinomas compared to alveolar epithelium ($p < 0.01$). Nuclear survivin expression was significantly higher than cytoplasmic survivin expression compared to the normal tissue ($p < 0.05$).

Discussion

The balance between apoptosis and anti-apoptosis signal pathways play an important role in determining the pathogenicity of several cancers. The said balance shifts towards anti-apoptosis in cancers, whereas it shifts towards apoptosis in tissues and organs of normal viability. Emerging clinical and animal models have gradually increased the studies aiming to shift the balance towards apoptosis. The fact that inhibition of apoptosis is one of the most

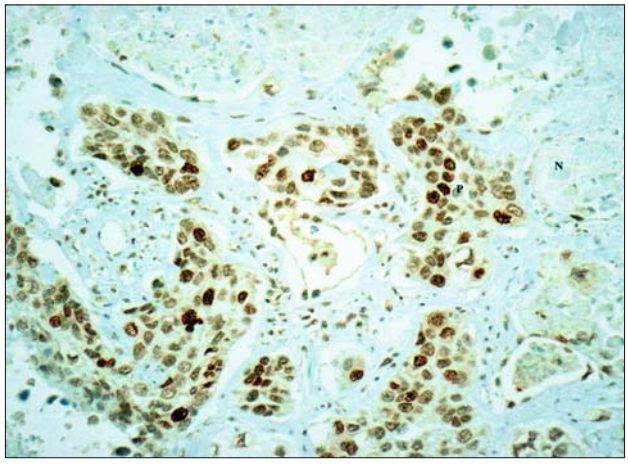

Fig. 3. Heterogenic-strong nuclear and few delicate shades of cytoplasmic survivin expression in squamous lung cell carcinomas. Immunohistochemical staining of survivin, x100.

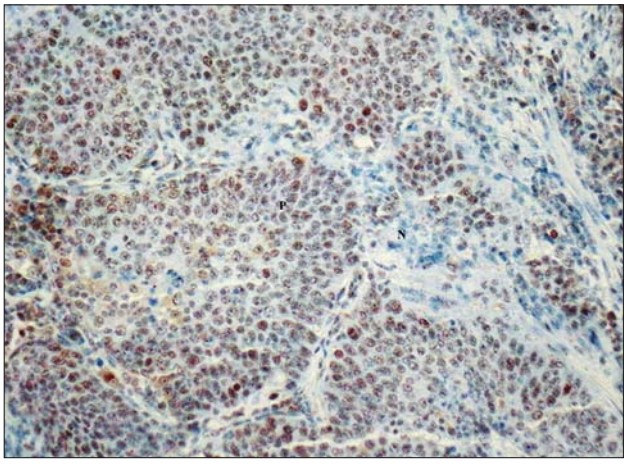

Fig. 4. Few delicate shades of nuclear and cytoplasmic survivin expression in alveolar epithelium. Immunohistochemical staining of Survivin, x100.

important goals in this regard makes the survivin gene and its protein one of the main targets in this field. The studies on this subject matter have focused on survivin with the understanding of the complex control mechanisms of survivin gene expression (7–10).

With the importance of the promoter region in survivin expression and the ability to detect epigenetic changes in this region, studies on silencing mechanisms associated with the promoter are currently ongoing. One of the major reasons of focusing these studies on survivin is the fact that presence of survivin expression does not lead directly to cancer; however, anti-cancer agents stimulate apoptosis-inducing mechanisms and apoptosis cannot be induced in the presence of survivin, which decreases the efficacy of cancer therapies. Successful silencing of the survivin mechanism in cancer cells would allow anti-cancer agents to be more effective and would probably change the course of treatment (11–15). Studies on methylation of the survivin gene have focused on the promoter region. There appears to be no studies on methylation of exon 1 of the survivin gene in literature. When we investigated exon 1 of the survivin gene by means of the analysis program, we found a potential region for epigenetic alteration. The present study is the only study investigating this region in literature. In summary, allowing the inhibition of survivin mechanisms would not be the treatment itself but would increase the efficacy of existing treatments, thereby offering an increased survival and quality of life in cancer patients. Furthermore, inadequate inhibition of survivin gene expression and activation mechanisms may also reduce the efficacy of future cancer therapies. Therefore, there are increasing attempts to investigate the epigenetic mechanisms, which activate the gene in question and change the amount of its expression as well as attempts to develop novel therapies targeting these epigenetic mechanisms. In the present study, during which we investigated methylation changes in exon 1 of the survivin gene, our patient group consisted of 41 cases with histologically confirmed squamous cell carcinoma tumours, and non-methylation was seen in all tumours and normal samples. Comparison of immunohistochemical detection of the survivin protein revealed a statistically significant increase (p: 0.01) in survivin protein, which had an inverse no-correlation with methylation rate during the transformation from normal to tumour tissue in exon 1 of survivin. The increased survivin protein in remaining tissues with a complete absence of methylation may be associated with the changes in complex control mechanisms of survivin expression as discussed above. Comparison of patients' survival revealed that they were alive except a few cases, and the finding that life expectancy has not been met in these patients may be explained with the fact that majority of these patients are cases who were diagnosed in early stages of their disease. However, we will continue to follow our patients in terms of survival for a few more years. As per the findings of our study; exon 1 of the survivin gene is not methylated in normal tissues or tumour tissues. The increased survivin protein expression in squamous tumours is not associated with the non-methylation status in exon 1 of the survivin gene; and we believe that it may be related to expression factors or promoter methylation, which may affect the survivin gene expression. Based on our findings, although the increased survivin protein expression in squamous tumours is not associated with the non-methylated condition of exon 1 of the survivin gene, we believe that it may be associated with the promoter methylation or expression factors, which may affect survivin gene expression. Furthermore, carcinoma formation after squamous epithelium replaces epithelial cells and the fact that survivin is an AIP as well as survivin positivity in both bronchial tissue and tumour cells may contribute to tumour development.

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