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Original Paper

Differential Binding of Nuclear Proteins to the *TP53* Gene Promoter in Male Breast Tumour

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It is well established that *TP53* regulates the expression of many genes, but the regulation of expression of *TP53* itself is poorly understood. Recently, it has been shown that there is a tissue-specific binding of nuclear proteins in the *TP53* gene promoter. The aim of this study was to determine the nuclear proteins that bind to the *TP53* promoter elements (between −104 and −458) in male breast cancer. The results of our study, using the electrophoretic mobility shift assay (EMSA) and Southwestern analysis, have showed: (1) nuclear proteins or factors other than p53 bind to the *TP53* promoter; (2) the levels of at least four nuclear proteins vary between normal and tumour breast tissue; and (3) two newly discovered nuclear proteins bind to the *TP53* promoter in tumour tissue but are absent in normal tissue. This differential binding of nuclear proteins to the *TP53* gene promoter might play a critical role in *TP53* transcription and cancer progression in male breast tumours. © 1997 Elsevier Science Ltd.

Key words: p53 promoter, EMSA, Southwestern, nuclear proteins, male breast tumour

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INTRODUCTION

TP53 MUTATIONS are found in a broad spectrum of human tumours [1–3] and affect control of cell growth [4]. Wild-type p53 blocks cell proliferation [5, 6] and can induce programmed cell death [7] in a variety of cellular systems. The versatile functions of p53 and current research in this field have been recently reviewed [8]. p53 is known to both activate [9, 10] and suppress [11, 12] transcriptional activity from various gene promoters. Thus, p53 acts as a general transcription factor regulating the expression of many genes. How the regulation of p53 occurs has not yet been determined. Transcriptional regulation of the *TP53* gene is controlled by two promoter elements. One promoter lies in intron 1, approximately 1000 bp downstream of the first p53 exon [13], while the other promoter is located 100–250 bp upstream of the non-coding first exon [14]. The *TP53* promoter lacks a TATA box and there is a CAAT box on the opposite strand at −80 with respect to the start site [15, 16]. However, it is not known whether this CAAT box is required for efficient promoter function. It has been shown that wild type *TP53* contains a p53 response element (between +22 and +67) and regulates its own transcription [17]. A basic helix–loop–helix binding motif (CACGTG) is present in an exon between +70 and +75 and the transcription factor USF binds to this bHLH motif to enhance the activity of the *TP53* promoter [18]. Furthermore, the levels of expression of mutant forms of p53 and p53 mRNA levels in some B-lymphoid cell lines correlate with cellular c-myc protein levels; c-myc–max heterodimers bind to the b-HLH site in the *TP53* promoter and activate it [19]. The *TP53* promoter region (−46 to −70) has been reported to be essential for *TP53* activation induced by genotoxic agents such as anticancer drugs and UV [20]. It has been shown that pax protein binds to the 5' region (+181 to +209) of the *TP53* gene in primary human diffuse astrocytomas and inhibits its expression [21]. Recently, it has also been shown that transcription factors YY1 and NF1 activate the human *TP53* promoter by alternatively binding to a composite element (−193 to −231) and E1A which cooperate to amplify *TP53* promoter activity [22]. Therefore, the differential regulation of these two promoters seems to be important in modulating expression of the *TP53* gene. Thus a clear picture is yet to emerge regarding the regulation of expression of the *TP53* gene.

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Cancer of the male breast is a rare disease accounting for less than 1% of cancer in males and representing less than 1% of all breast cancers [23]. The biology and aetiology of the disease are very poorly understood. Recently, tissue-specific binding of nuclear proteins to the *TP*53 promoter has been demonstrated [22]. The *TP*53 promoter region from −104 to −458 is sufficient for full promoter activity [14]. Hence, an attempt has been made to analyse the binding of specific nuclear proteins, if any, with the *TP*53 promoter elements between −104 to −458 during the process of tumorigenesis in the male breast.

MATERIALS AND METHODS

Tissue samples

Tumour tissues were collected from the A.H. Regional Centre for Cancer Research and Treatment, Cuttack, India, on the day of surgery. Control tissues were collected form uninvolved adjacent areas of the breasts. Both control and tumour tissues were examined histopathologically. The confirmed malignant samples were selected for further studies.

Preparation of nuclear extract

Nuclear extracts were prepared from normal and tumour samples according to the method of Costa and associates [24] with slight modification. Tissues were ground up in liquid nitrogen and homogenised in homogenisation (H) buffer (2g/4 ml) containing 0.3% Triton X-100 at 4°C (H buffer contains 10mM Hepes (pH 7.6), 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1mM EGTA, 1mM EDTA, 0.5 M sucrose, 1 mM dithiothreitol, 0.5 mM phenylmethysulphonyl fluoride, aprotinin at 2 μg/ml, pepstatin at 0.7 μg/ml and leupeptin at 0.7 μg/ml). This was filtered through cheesecloth and centrifuged at 3000 ***g*** for 10 min. Nuclei were resuspended and repelleted twice more, once with and once without Triton X-100. The final pellet was resuspended in 1 ml H buffer and lysed with 0.8 ml H buffer lacking sucrose but containing 15% (vol/vol) glycerol and 2 M KCl (final KCl concentration, 0.8 M). Nuclear proteins were extracted at 4°C with gentle agitation for 1 h, chromatin was pelleted, and the supernatant was dialysed for 5 h, against a 100-fold excess of 0.1 M KCl/20 mM Hepes, pH 7.5/0.1 mM EDTA/10% glycerol (two changes) and clarified by centrifugation at 10000 ***g*** for 20 min. Nuclear extracts were quantified by the Lowry method [25], aliquoted and stored at −70°C.

Preparation of the DNA probe

The probe used in EMSA and Southwestern analysis was generated by cutting the *TP*53 promoter with appropriate restriction enzymes (Figure 1). The *TP*53 promoter fragment from −104 to −458 (Xba 1–Bam H1) was purified on agarose gel and end-labelled with DNA polymerase 1 klenow fragment [26].

Electrophoretic mobility shift assay (EMSA)

Binding of nuclear proteins to the *TP*53 promoter has been studied by EMSA [27]. Binding reactions were done in a 20 μl volume containing 10 mM Hepes (pH 7.6), 50 mM KCl, 1.5 M $MgCl_2$, 10% glycerol, 1 mM dithiothreitol, 1 μg *E. coli* DNA and nuclear extract. Samples were pre-incubated at room temperature for 5 min before the addition of the radiolabelled DNA probe (5000 c.p.m.). In supershift EMSA, 1 μg of p53 antibody (purchased from Santa Cruz Biotech, California, U.S.A.) was added in the binding reactions. The binding was allowed to proceed at room temperature for 20 min. Samples were then electrophoresed through 4% non-denaturing polyacrylamide gel containing 0.04% bisacrylamide, 5% glycerol and 0.5X Tris-Borate-EDTA [26]. Gels were then autoradiographed.

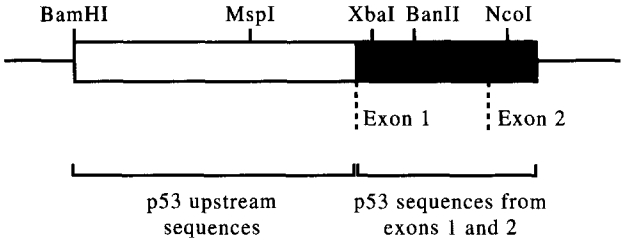

Figure 1. Restriction map of the *TP*53 promoter region, exon 1 and part of exon 2.

Southwestern (DNA–protein) blotting

Southwestern (DNA–protein blotting) analysis was done using the Xbal-BamHl end-labelled fragment [26, 28]. The volume and molecular weight of proteins were determined using Image Quant Programme of a Scanning Densitometer (Molecular Dynamics, California, U.S.A.). Nuclear extracts (20 μg) were electrophoresed through 8% SDS–polyacrylamide gel and blotted onto nitrocellulose membrane. The membrane was incubated in binding buffer (same as EMSA) containing 5% Denhardt's reagent for 2 h and then in the same buffer containing *E. coli* DNA (50 μg/ml) and 1×10^6 c.p.m/ml ^{32}P-labelled Xba 1-Bam Hl fragment

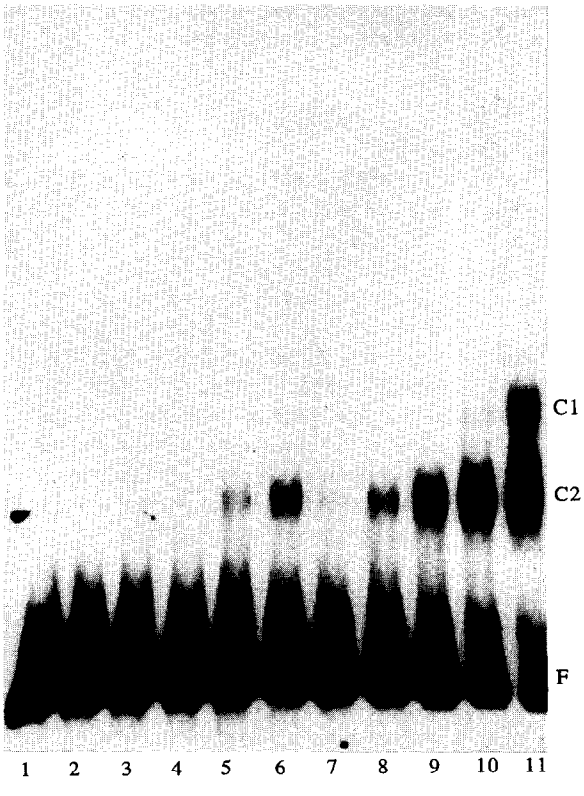

Figure 2. EMSA analysis of the *TP*53 promoter (Xba 1-Bam Hl fragment). Lane 1, free probe, lanes 2, 3, 4, 5 and 6 normal sample containing 0.5, 1, 2, 4 and 8 μg of nuclear extract respectively; lanes 7, 8, 9, 10 and 11, tumour sample containing 0.5, 1, 2, 4 and 8 μg of nuclear extract, respectively.

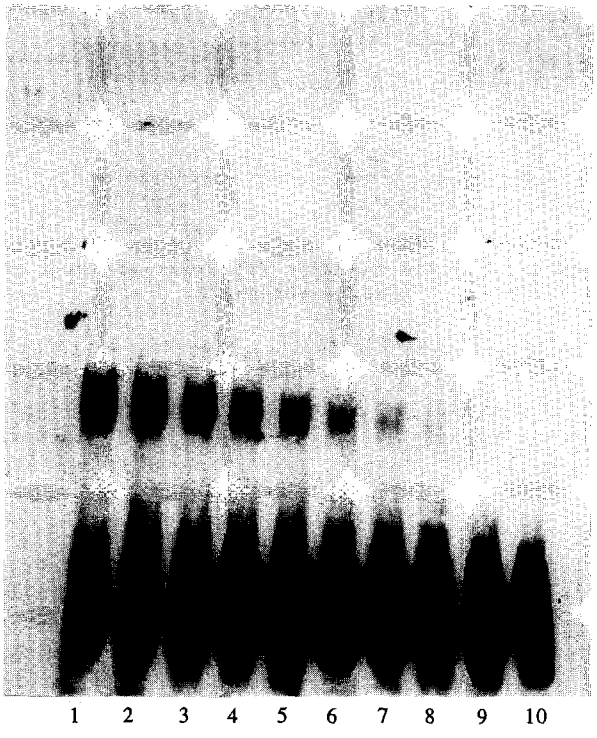

Figure 3. Disappearance of protein–DNA complexes with unlabelled *TP*53 promoter. Lane 1, labelled probe + nuclear extract (normal); lanes 2, 3, 4, 5, 6, 7, 8, 9 and 10, probe + - nuclear extract (normal) + unlabelled probe (0.1, 0.2, 0.4, 0.8. 1.6, 3.2, 6.4, 12.8 and 25.6 ng, respectively).

(−104 to −458) for 2 h at room temperature. After washing three times in binding buffer for 10 min each at room temperature, the membrane was autoradiographed.

RESULTS

EMSA of p53 promoter

The results of EMSA have shown one protein–DNA complex in normal and two complexes in tumour breast tissue (Figure 2). However, the level of nuclear proteins is greater in tumour tissue than that of its normal counterpart. However, the second protein–DNA complex also appears in normal tissue on increasing the concentration of nuclear extract (data not shown). Supershift EMSA was performed by using p53 antibody. Addition of anti-p53 antibody in the binding reactions did not result in a supershift band (data not shown). Further, cold titration was performed using unlabelled promoter fragment in the binding reactions. As the concentrations of unlabelled probe increased, the protein–DNA complex gradually disappeared (Figure 3).

Southwestern analysis of nuclear proteins

The number of proteins which bind to the *TP*53 promoter element was studied by Southwestern blotting. In normal tissue, at least four proteins (approximately 131 kDa, 121 kDa, 112 kDa and 51 kDa) bound to the *TP*53 promoter. All these four proteins are also present in tumour tissue but their level varied (Figure 4). However, three among these four proteins, i.e. 131 kDa, 121 kDa and 112 kDa, were not distinct and appeared as one band in the photograph, although they were quite distinct in the autoradiogram, and this was also confirmed by scanning densitometry. In tumour tissue, the level of the 131 kDa

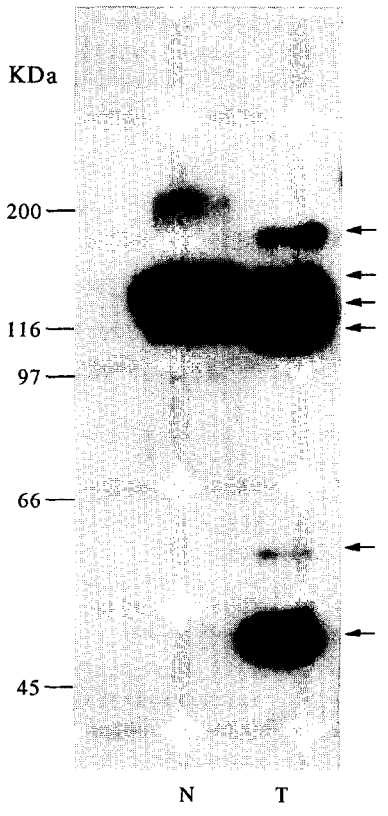

Figure 4. Southwestern blot of nuclear proteins. Lane N, normal tissue, lane T, tumour tissue. Protein markers are indicated in kDa. Arrowheads indicate different nuclear proteins.

protein was 4-fold more, the 121 kDa protein was 1.3-fold less. The 112 kDa protein was 5-fold more and the 51 kDa protein was approximately 60-fold more compared with levels in normal tissue. In addition to these four proteins, at least two new proteins (approximately 176 kDa and 60 kDa) appeared in tumour tissue but were absent in the normal tissue.

DISCUSSION

Our results of EMSA indicate an increased binding pattern of nuclear proteins to *TP*53 promoter in tumour tissues compared with that of their normal counterparts. In view of the report by Deffie and associates [17], p53 itself seems to be involved in *TP*53 gene expression. We therefore examined the possible binding of p53 to its promoter elements in a supershift EMSA using p53 antibody. Supershift EMSA did not show any supershift band, which suggests that nuclear proteins other than p53 bind to this promoter element. Cold titration experiments using unlabelled *TP*53 promoter suggest that binding of nuclear proteins to the *TP*53 promoter element is specific.

We showed by Southwestern analysis that the level of three proteins binding to the *TP*53 promoter element increased and also that two other new proteins appeared in the tumour tissue. The 51 kDa protein was present in an almost undectable level in normal tissue, whereas the level was approximately 60-fold higher in tumour tissue. Such a differential binding of nuclear proteins to the *TP*53 promoter in normal and tumour tissue might be responsible for transcriptional deregulation of the *TP*53 gene. It has been

shown that elevated levels of USF [18] and YY1 and NF1 [30] activate *TP53*, whilst tax oncoprotein [29] and pax protein [21] repress transcription of *TP53*. Therefore, both transcriptional activation and repression of the *TP53* gene are associated with tumorigenesis or neoplastic transformation. In principle, during oncogenic transformation either deregulated expression of transcription factors or mutations in the *TP53* promoter, resulting in enhanced protein binding, could lead to altered expression of p53 [18]. Recently, it has been shown that binding of transcription factors YY1 and NF1 to the *TP53* promoter (−193 to −231) varies in a tissue-specific manner. YY1 binds to this element in nuclear extracts of rat testis and spleen and NF1 in extracts of liver and prostrate [22]. This may facilitate tissue-specific control of *TP53* expression [22]. Our results indicate that there is a differential binding of nuclear proteins to the *TP53* promoter in normal and tumour tissues, and it remains to be determined whether these factors activate or repress *TP53* transcription. Our previous results have shown an overexpression of p53 protein in male breast tumours [30]. Sufficient fresh tissues were not available for p53 mRNA analysis. However, these nuclear factors might be playing a critical role in the process of tumorigenesis of male breast, but this is a very preliminary observation. Work is in progress to synthesise shorter *TP53* promoter fragments which will be used in EMSA to determine the specific promoter elements involved in the binding of nuclear proteins. Further characterisation of these proteins might help in the better understanding of *TP53* regulation and cancer progression.

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