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Serum and Urinary Vascular Endothelial Growth Factor Levels in Non-small Cell Lung Cancer Patients

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INDUCTION OF new capillary blood vessels is required for tumour growth [1]. At the beginning of their development tumours are not vascularised and the induction of an angiogenic phenotype switches tumours to a more aggressive behaviour. Angiogenesis is mediated by specific angiogenic peptides (basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), tumour necrosis factor-α (TNFα)) probably released by tumour cells and tumour-associated macrophages [2–4]. The angiogenic peptides are mitogenic for several tissues, with the exception of VEGF, which is specific for endothelial cells [5]. VEGF is also a potent vascular permeabilising agent which causes leakage of plasma proteins with formation of a provisional stroma that allows the migration of endothelial cells and fibroblasts. VEGF is a 34–45 kDa protein which exists in four different isoforms of 121, 165, 189 or 206 amino acids. It is expressed in many normal tissues and is overexpressed in many non-neoplastic diseases (psoriasis, rheumatoid arthritis and ischaemic heart disease) [6]. Together with its two receptors (flk-1 and KDR), VEGF is highly expressed both at the mRNA and protein level in tumour cells and in endothelial cells of new borne vessels [7].

In several studies, raised serum levels of angiogenic peptides are associated with poor prognosis [8]. In order to study the relationship between VEGF levels in biological fluids and non-small cell lung cancer (NSCLC), we measured VEGF levels in serum and urine of 40 patients (mean age ± S.D., 62.5 ± 8.5 years) with newly diagnosed NSCLC (stage I:10; stage II:8; stage IIIA:8; stage IIIB:7; stage IV:7) and in 18 healthy controls (mean age ± S.D., 40 ± 12 years).

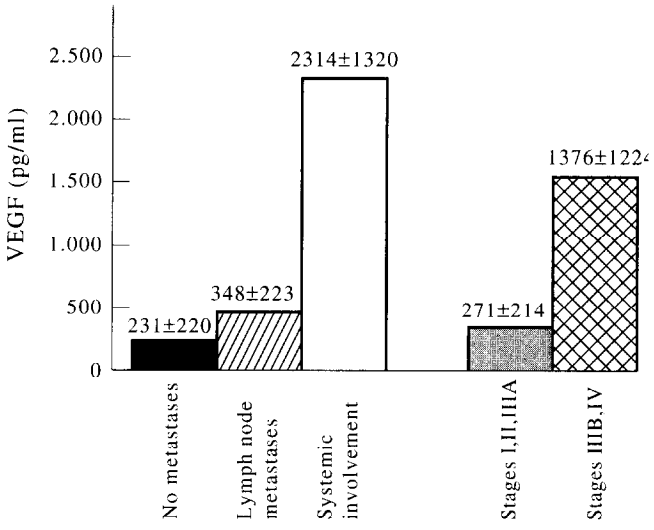

Figure 1. Relationship between serum VEGF and extent of disease.

Serum samples were collected by venipuncture, centrifuged and then stored at −70°C until assayed. VEGF levels in serum and in urine were determined using an immunoenzymatic assay where samples and biotinylated cytokine compete for the same antibody binding site. Measurements were made at a wavelength of 492 nm. Data are expressed as mean ± standard deviation (S.D.), and were statistically analysed using the Student's *t*-test. P values < 0.05 were considered statistically significant. The mean value of serum VEGF in healthy controls was 66.01 ± 96.22 pg/ml, with a significant difference between males (12.50 ± 6.53 pg/ml) and females (133.35 ± 114.7 pg/ml) ($P = 0.036$). In NSCLC patients, serum VEGF ranged from 38.96 to 4275 pg/ml and the mean value (602 ± 847.29 pg/ml) was significantly higher than mean VEGF values of healthy controls ($P = 0.01$). We considered 'raised' VEGF levels those ⩾ the mean + 3 S.D. of healthy controls (355 pg/ml); the percentage of patients with 'raised' VEGF increased from 39% for stage I to 100% in stage IV (50% in stage II, 62.5% in stage IIIA and 86% in stage IIIB). In the whole group, patients with systemic involvement (2315 ± 1320 pg/ml) showed a significantly higher mean serum level than patients without lymph node or systemic involvement (231.06 ± 220.73 pg/ml; $P < 0.001$). Although patients with lymph node involvement showed a higher mean value (348.01 ± 223.80 pg/ml) than patients without lymph node involvement, the difference was not statistically significant (231.06 ± 220.73 pg/ml, $P = 0.135$). Moreover, patients with advanced disease (stage IIIB and IV) (1376.08 ± 1224.84 pg/ml) had a higher mean value than that of early stage patients (271.03 ± 214.22 pg/ml) ($P < 0.001$) (Figure 1). Urinary level in healthy controls was 1.226 pg/ml and 2.26 pg/ml in NSCLC patients (P = n.s.); a statistical difference was found only between normal subjects and patients with stage IV disease (4.52 ± 2.59 pg/ml; $P = 0.010$).

In conclusion, VEGF serum levels appear to be increased in patients with NSCLC; these preliminary data show a re-

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lationship between raised serum VEGF levels and the extent of the disease. Further studies are warranted to assess the prognostic value of this peptide in lung cancer patients, as well as its ability, together with microvessel count [9], to predict the risk of metastasis.

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Abnormalities of the *P16^{INK4A}* Gene in Thyroid Cancer Cell Lines

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LOSS OF tumour suppressor gene activity is a key event in the genesis of most human tumour types. Predictably, some of these genes code for negative regulators of the cell cycle. Over the last 2 years, one of this group, the cyclin kinase inhibitor p16INK4a has been found to be abnormal in a wide range of primary cancers and tumour-derived cell lines [1]. Recently, we reported the first study of thyroid cancers in which four of a series of seven showed deletion of the *p16* gene locus [2]. In the light of recent reports pointing to the occurrence of point mutations [1] and differences in transcriptional regulation [3], we have now looked for more subtle lesions in the remaining cell lines that appeared to have a normal *p16* gene in our initial report, namely, FTC 133 (derived from follicular carcinoma), BCPAP and NPA (both from papillary cancers). Two types of analyses were performed; sequencing of genomic DNA and methylation status.

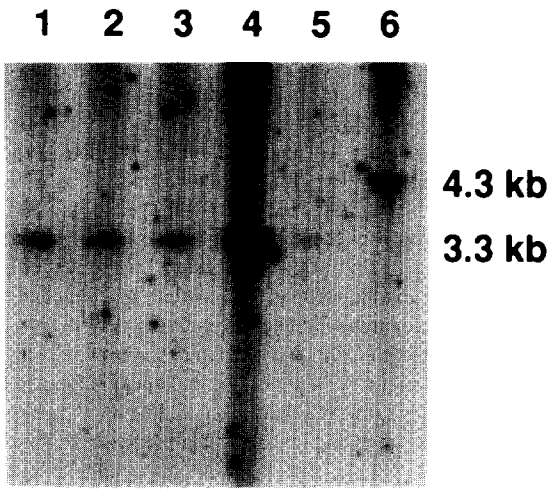

Figure 1. DNA digested with Sac II and EcoRI was probed with *p16* exon 1 after transfer to a Hybond N+ membrane. The probe hybridises to unmethylated and methylated exon 1 at 3.3 and 4.3 kb, respectively. Lanes: (1) placental tissue; (2) normal adult thyroid tissue; (3) thyroid cells prepared from Graves' disease (non-neoplastic); (4) ori 3 (an SV40 transformed epithelial cell line not expected to show *p16* abnormality); (5) FTC133 (follicular cancer cell line); (6) BCPAP (papillary cancer cell line).

In sequencing the genomic DNA, the two exons in which most of the *p16* gene (approximately 97%) lies, were isolated by PCR (polymerase chain reaction) using primers as follows: exon 1 GAAGAAAGAGGAGGGGCTG and GCGCTACCTGATTCCAATTC; exon 2 ACACAAGCTTCCTTTCCGTC and TCTGAGCTTTGGAAGCTC. Genomic DNA (100 ng) was amplified for 40 cycles (94°C, 40 s; 60°C, 40 s; 72°C, 90 s) under standard conditions with the addition of 3.6% formamide. Purified PCR products were subcloned using the TA system (Invitrogen). Both strands of three clones from each cell line were analysed by double-stranded DNA cycle sequencing on an ABI 373 system using the original PCR primers, ABI Prism dye terminators and Taq FS.

In the NPA cell line, a point mutation in the splice donor consensus site flanking exon 1, was identified (T → C at base 256, Genbank U12818). This would result in an incorrectly spliced mRNA and inappropriate translation, effectively abolishing *p16* function. This observation was confirmed by direct sequencing of another PCR product from NPA DNA. The other cell lines had wild type *p16* sequence.

Reduction of gene expression, caused by *de novo* methylation of CpG islands within exon 1 of *p16*, has been identified in both primary tumours and tumour-derived cell lines [3].

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