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CIRCULATING DBP LEVEL AND PROGNOSIS IN OPERATED LUNG CANCER: AN EXPLORATION OF PATHOPHYSIOLOGY

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ABSTRACT

Background: Vitamin D stimulates transcription of anti-angiogenic and apoptotic factors that may suppress tumours, whilst vitamin D binding protein (DBP) may be a biomarker in murine lung cancer models. We sought to ascertain if the vitamin D axis is altered in lung cancer or influences prognosis.

Methods: 148 lung cancer patients, 68 other intrathoracic cancer patients and 33 non-cancer controls were studied for up to 5 years. Circulating DBP and vitamin D levels were compared between groups and their effect on survival assessed by Cox regression analysis. Expression of DBP and vitamin D receptor (VDR) was examined in lung cancer cell lines and in normal and tumour lung tissue by Western blot and immunohistochemistry.

Results: Low serum DBP levels predicted lung cancer specific death ($p=0.04$), and DBP was poorly expressed in lung cancer cells on Western blot and immunohistochemistry. Vitamin D did not predict cancer survival, and VDR expression was variable in tumours.

Conclusions: Preservation of serum DBP is a significant independent factor associated with better cancer outcome in operated lung cancer patients. Given the established role of DBP in macrophage activation and clearance of abnormal cells further study on its involvement in lung cancer is merited.

INTRODUCTION

Vitamin D is a fat soluble vitamin best known for its role in calcium and phosphate homeostasis. It is also increasingly apparent that Vitamin D has beneficial health effects beyond the skeletal system. Serum concentration of cholecalciferol (vitamin D3; $25OHD_3$) is the best indicator of vitamin D status as it reflects cutaneous production as well as that intake in foods and supplements, whereas 1, 25-Dihydroxycholecalciferol ($1,25\ (OH)_2D_3$) has a short half life and serum concentrations are tightly regulated [1]. Vitamin D status has been reported to correlate with cancer risk, and play a role in the prevention of colon, prostate and breast cancers [2], although less is known about its influence on lung cancer. None of the available epidemiological work has been able to determine the level of risk conferred by vitamin D deficiency, because of confounders such as obesity and amount of sunlight exposure.

Vitamin D may suppress tumour progression by reducing cell proliferation, invasiveness and angiogenesis, and stimulating apoptosis [2-4]. It also protects against metastases in various tumour models, including the lung [2-4]. In order for vitamin D to exert its intracellular effects it must enter cells by diffusion or by endocytosis when bound to its main carrier protein, vitamin D binding protein (DBP). When intracellular, vitamin D is dissociated from DBP and then undergoes a series of reactions that enable interaction with the vitamin D receptor (VDR) – a process illustrated in our previous work[5]. There is some suggestion that VDR expression is reduced in lung cancer [6], implying that vitamin D will be less able to exert its anti-tumour effects, such that other aspects of the vitamin D axis could be more important.

DBP is a glycosylated alpha globulin, part of the albumin superfamily, being about 58kDa in size, produced in the liver and located predominantly in serum. It is divided into 2 large domains (I and II) and a shorter domain at the COOH terminus (domain III) [7], and is expressed in many tissues [8] and by neutrophils [9]. It contributes to macrophage activation [10], augments monocyte and neutrophil chemotaxis to C5-derived peptides and acts as an actin scavenger protein, as discussed in our recent review [5]. It may play a role in malignancy

because of its effect on macrophages, which are important because of their potential to clear abnormal tissue [11]. Indeed in lung cancer, the number of cytotoxic macrophages within the tumour predicts survival [12]. The vitamin D axis may optimise anti-tumour actions of macrophages in two ways. Firstly, DBP can be converted by de-glycosylation to a potent macrophage activating factor (DBP-MAF) [10]. Thus in tissue where DBP is poorly expressed, or poorly converted to DBP-MAF, macrophage activation will be sub-optimal. Little is known about DBP expression in lung tissue, although we have demonstrated previously that DBP is present in airway secretions [13]. Secondly, macrophages can convert $25OHD_3$ to 1,25 $(OH)_2D_3$ [14], thus optimizing downstream effects on anti-tumour gene transcription in these cells.

We hypothesized that the vitamin D axis may be altered in lung cancer and relate adversely to prognosis: this may be due to either vitamin D deficiency, inability of tumour tissue to respond to vitamin D, or reduced macrophage activation by DBP-MAF in and around tumours.

METHODS

Prognostic effect of serum markers of the vitamin D axis

Patients were recruited consecutively from thoracic surgery lists at Heart of England NHS Foundation Trust (HEFT) between 2006 and 2009. Serum samples were taken at several time points, as part of the CLUB study, a prospective study of potential lung cancer biomarkers which is described previously[15]. The current project was a sub-study, and only those with pre operative samples remaining were selected. This gave a total of 148 lung cancer patients, 68 other intra thoracic tumours and 33 non-cancer controls. Demographic features, tumour histology and pathological stage, surgery type, resection margins, smoke exposure and co-morbidity were recorded. Pathological staging was taken to be the gold standard and has been updated to reflect the latest staging guidance for non-small cell lung cancer (NSCLC)[16]. Lung cancer patients were followed for up to 5 years and survival assessed using Cancer Intelligence data. The study was approved by the local ethics committee and all patients gave informed

consent. Vitamin D ($25OHD_3$) was measured by tandem mass spectrometry at HEFT. DBP was measured by specific ELISA (Immunodiagnostik).

Assessment of VDR and DBP in lung cancers and normal lung

Tumour and non-tumour lung tissue was obtained from 25 patients undergoing resection at HEFT between 2009 and 2010. After resection lungs were taken immediately to the pathology department for inflation with 10% formalin at constant 25cm H_2O pressure via cannulation of the major airway, and once inflated, were immersed in formalin for 24 hours. Representative blocks of normal lung distant from the tumour and tumour blocks were selected by a single pathologist for subsequent staining. Both VDR and DBP were stained using the Ventana Benchmark XT system with ultraview technology. The primary mouse monoclonal antibodies used were anti-VDR (D-6): SC-13133 (Santa Cruz Biotechnology Inc, USA), and anti-DBP A0021 (Dako, UK). Staining with both antibodies involved a 30 minute CC1 antibody retrieval step, followed by 32 minutes antibody incubation. The VDR antibody was diluted 1:100 and DBP 1:10000. An additional 4 minute haematoxylin counterstain was used in the anti-DBP protocol. The VDR protocol was adapted from that published for lung tissue [6] and the DBP protocol from that published for kidney tissue [17]. Positive controls were kidney tissue (VDR) and liver (DBP) respectively; positive staining was determined by a pathologist using standard semi-quantitative techniques which grade intensity of staining[18].

Assessment of VDR and DBP in lung cancer cell lines and normal lung tissue

Cancer cell lines were described in our previous work and cultured as indicated therein [19]. NCI-231 was originally gifted to IT by Dr Y Shimosata of the National Cancer Research Institute in Japan in 2003. A549s, NCI-H292 and NCI-H69 were purchased from the American Type Culture Collection, USA, and authenticated at source in 2003. Lu65 and Lu99 were purchased from the Riken Bioresource Cell Center Japan, and authenticated at source in 2003. HARA were purchased from the Health Sciences Research Resource Center, Japan, again in 2003. All cells were tested prior to the experiments herein for N-CAM expression by flow cytometry (either

positive or negative – data not shown) and Mycoplasma (all negative), as described in our previous work [19].

Cells were lysed in a buffer containing 1% Triton X-100, 0.1% SDS, 1mM EDTA, 10mM Tris/HCl, pH 7.5, 150mM NaCl, 0.01% sodium azide and a protease inhibitor cocktail (Sigma). The protein concentrations of lysates were determined using the Detergent Compatible Protein Assay (Bio-Rad), and 45μg reducing samples were separated on SDS-polyacrylamide gels. Protein was transferred to PVDF membranes and probed with chicken anti-DBP, rabbit anti-VDR or mouse anti-tubulin antibodies (all Sigma). For DBP and tubulin blots, secondary antibodies were IRDye 800CW-conjugated for visualization using the Odyssey Infrared Imaging System (LI-COR). For VDR blots, the secondary antibody was horse-radish peroxidase-conjugated (Thermo Scientific) for visualization using Pierce ECL chemiluminescence reagents (Thermo Scientific) and Hyperfilm (Amersham Biosciences), which was developed using a film processor (AGFA Curix 60).

Statistical analysis

All analyses were carried out in SPSS version 16.0 (Chicago, USA). Clinical data normality was assessed using the Kolmogorov-Smirnov test (normal, $p>0.05$); parametric data is reported as mean (SEM) and non-parametric data as median (range). The t-test was used to compare means of parametric data and the Mann Whitney or Kruskal Wallis for non-parametric data between groups. Frequency variables were compared using the Chi squared test. Bonferroni correction for multiple tests was used for these analyses meaning that unadjusted overall p for significance was 0.01. A multivariate Cox regression analysis was carried out for survival of those NSCLC cases with clear resection margins using age, gender, smoke exposure, histological type and cancer stage, plus DBP or vitamin D level as predictors. DBP was assessed in quartiles (4 groups), rather than as a continuous variable. All comparisons of vitamin D took into account season of collection, as described in our previous work [13]. Statistical significance was assumed at $p<0.05$ in the absence of Bonferroni correction.

RESULTS

Prognostic effect of serum markers of the vitamin D axis

Characteristics of the patients are shown in table 1. None were taking prescribed vitamin D supplements when admitted for surgery. There were no significant differences between the two cancer groups (all $p>0.05$). Non-cancer controls were younger, more likely to be male and had been followed up for fewer years ($p=0.03$, <0.01 and 0.02 respectively).

	Lung cancer N=148	Other cancer N=68	Non-cancer controls N=33
Age	69.7 (1.5)	66.6 (54-80)	54.5 (33-88)
Male gender	57 (38.5)	22 (32.4)	24 (72.7)
Pack years smoked	50.0 (5-120)	60.0 (30-100)	43.3 (0-70)
Current smoker	57 (38.5)	9 (13.2)	1 (3.0)
Never smoked	7 (4.7)	3 (4.4)	4 (12.1)
Cancer death	33 (22.3)	-	-
Other death	23 (15.5)	-	-
Years of follow up	4.3 (1.5-5.3)	4.5 (1.8-5.7)	2.38 (1.4-5.0)
DBP (mg/dl)	33.7 (1.5)	35.9 (2.4)	45.5 (5.1)
Cholecalciferol (ng/ml)	38.5 (1.6)	15.7 (2.0)	30.8 (3.0)
Albumin (g/l)	37.6 (3.8)	33.4 (27.2-39.6)	41.3 (2.7)

Table 1: Characteristics of the patients

Frequency data is shown in bold types and is listed n (%). For the quantitative data, where it was normally distributed mean (SE) is shown and for non-normal data median (range).

The histology of the lung tumours, and pathology of the other patient groups is shown in Figure 1. Squamous cell carcinomas were the most frequent lung tumour, whilst oesophageal cancers

formed the majority of the other cancers. 6 patients had small cell lung cancer and were excluded from further analyses. Amongst the NSCLC cases pathological stages were distributed as follows : Ia=27 patients; Ib=45; IIa=11; IIb= 10; IIIa=22; IIIb=7. In 20 cases new staging could not be determined from the pathology report due to the level of detail given. Amongst the other cancers all oesophageal patients were stage IIa or IIb, all mesothelioma patients were stage II and of the 2 lymphoma patients one was stage II and one stage III.

Cholecalciferol varied with season of collection, as expected, although this difference was marginal and unlikely to be of clinical significance (Supplementary data). After adjustment for this it tended to be higher in lung cancer than non-cancerous lung disease, although this was not statistically significant (38.5 v 30.8 ng/ml; p=0.06). In other cancers vitamin D was lower (15.7ng/ml; p<0.01). This is shown in Figure 2a. Frequencies of the 3 usual classes of vitamin D level in the lung cancer patients are shown in Figure 2b. DBP was lower in lung cancer patients than non-cancerous lung disease (33.7 v 45.5 mg/dl; p=0.02) but did not differ from other cancers (35.9mg/dl; p=0.72). This is also shown in Figure 2a. DBP and cholecalciferol did not correlate with one another (p=0.62). Albumin did not vary significantly between groups (both p>0.32); there was no significant correlation between this and cholecalciferol (p=0.72) or DBP (p=0.24).

All patients had undergone at least 12 months of follow up at the time of data analysis. One year survival was 79.1%. Of those that died during their follow up period the median time to death was 0.93 years (range 0-3.54 years); when only cancer related deaths were selected mean time to death was 1.04 years (range 0-3.45 years). Survival at the mean of covariates, excluding DBP and vitamin D, is shown in Figure 3a, sub-stratified for quartiles of DBP in Figure 3b. Stage, age and pack years smoked were all significant predictors of death (p=0.039, 0.005 and 0.009 respectively), whilst gender was not (p=0.96). In the all cause mortality analysis neither DBP nor $25OHD_3$ predicted death (both p>0.30). When only deaths secondary to lung cancer were considered DBP became a predictor (p=0.041), the odds ratio of death falling to 0.95 (0.91-0.98)

for each unit gained in DBP. To put this into context, a lung cancer patient exhibiting DBP level equivalent to that of our healthy cohort would have an OR of death of 0.59 compared to a patient with the mean DBP level seen in our cohort. Further details of the DBP analyses are shown in table 2; the wide CIs reflect the small numbers of deaths.

DBP quartile	DBP range (mg/dl)	Deaths (n/total)	OR (95% CI)	p
4	>43.02	3/32	-	-
3	33.25-43.01	9/35	5.49 (0.62-48.52)	0.125
2	19.95-33.24	7/31	5.77 (0.59-56.64)	0.133
1	<19.94	7/34	10.4 (1.03-125.42)	0.044

Table 2: Relationship of quartiles of DBP to lung cancer specific death in the NSCLC cases

The table shows the range of DBP values in each quartile (numbered in descending order, such that 4 is the highest), the number in each quartile who died a lung cancer specific death(n)/number of individuals in that quartile, and the odds ratio for mortality compared to the highest quartile.

Albumin was also assessed as a predictor in order to ascertain if the DBP effect was specific; albumin was not significant (p=0.38). Cholecalciferol did not predict lung cancer death (p=0.52).

Assessment of VDR and DBP in lung cancers and normal lung

In normal lung VDR was expressed most strongly in bronchial epithelium, with lesser staining in pneumocytes (Figure 4a). Only one tumour exhibited absent VDR expression, however half exhibited less intense staining than the normal lung tissue from that individual (Figure 4b). In normal lung DBP was present predominantly in blood and airway secretions with less intense

staining in macrophages (Figure 4c). In general tumour tissue only stained positive for DBP in necrotic areas and associated macrophages, elsewhere it exhibited intensity half that of airway secretions (Figure 4d). 16% of tumours showed no DBP expression. Consistent with relatively low expression of VDR and DBP in lung tumours, eight lung cancer cell lines exhibited low or absent expression of these proteins by western blotting, compared with positive control blots of four normal lung samples (Figure 5). Normal lung was used a positive control because of the detection of these proteins in lung sections (Figure 4).

DISCUSSION

We have shown that low serum DBP before surgery may be a predictor of subsequent death from lung cancer, and that expression of DBP is either low or absent in lung cancer tissue. This supports a pathogenic role for DBP in lung cancer, which is most likely to centre on its role as a precursor for DBP-MAF, based on its location on macrophages in normal lung, and in necrotic areas in tumours. It seems likely that DBP is not produced extensively by lung tissue, but diffuses from blood to airway secretions and tissue fluids given that little staining was observed in any primary pulmonary cells in the normal lung samples, and the Western blots from cell lines showed no expression. This may explain why a serum marker was capable of predicting a lung specific outcome. Small amounts of DBP expression by normal lung and tumours remains a possibility. Prognostic markers in lung cancer include ERCC1, EGFR, RRM1 and KRAS, although most of these are better validated as markers in tumour than circulating blood [20]. A recent review of lung cancer biomarkers noted that many of the studies looking at such biomarkers and survival have been conducted retrospectively on samples collected during clinical trials, such that their role in predicting response to therapy rather than outcome per se is better known [20]. The hazard ratio for the lowest quartile of DBP was similar to that conferred by high levels of circulating cancer cells in a recent study of 101 patients with stage III or IV NSCLC [21].

The link between DBP and lung cancer has not been studied in detail; one study of circulating DBP levels showed no difference between cancer and healthy individuals [22]. However the techniques for measuring DBP used in this study were much less sensitive than the current ELISA, and the study itself was not specific to lung cancer, comprising a total of 100 cases, split between lung, prostate and gastrointestinal malignancies. More recently, proteomic work in a mouse model of lung cancer suggested that DBP acts as a disease biomarker [23]. DBP is regulated at a transcriptional level by pro-inflammatory cytokines and steroids[24], and could potentially relate to nutrition and catabolic states, rather like albumin, since it is in the same family of proteins. We did not show any relationship of survival to albumin levels, but cannot exclude an epiphenomenon linking DBP to another unmeasured poor prognostic factor influencing our DBP mortality analyses.

Previous work has shown that conversion of DBP to DBP-MAF may be reduced in malignancy due to the action of α-N-acetylgalactosaminidase [25]. During tumour invasion various cells in cancerous tissues produce exo- and endoglycosidases [26] and if the latter enter the bloodstream they are capable of deglycosylating circulating DBP, a process which appears to relate directly to tumour burden in a murine model [27]. Our data shows that DBP is low in the blood of lung cancer patients. Thus even if DBP deglycosylation is not involved, macrophage activation may be lower, adversely affecting prognosis. Augmentation of DBP-MAF has been proposed as adjuvant therapy in surgically resected cancers for these reasons; indeed in colonic and prostate cancers DBP-MAF immunotherapy used in this way was safe and well tolerated in early phase trials [28-29]. DBP-MAF has also shown beneficial effects on breast cancer cells in vitro [30]. These concepts require further follow up before trials of DBP-MAF would be appropriate in lung cancer, but provide an exciting new avenue for research. Specifically, a more extensive analysis on the expression of DBP-MAF and the mechanisms of deglycosylation in lung tissue would be required in the future.

Cholecalciferol did not predict outcome in our survival analysis. Few studies have been done examining vitamin D status specifically in lung cancer. Zhou et al. investigated the association between surgery season and vitamin D intake with recurrence-free survival (RFS) and overall survival (OS) in 456 early-stage NSCLC patients. They concluded that the joint effect of season and intake are associated and higher $25OHD_3$ levels correlated with improved OS and RFS [31]. In our study levels were higher in summer, although the differences were unlikely to be clinically significant; a specific survival analysis according to season of surgery was not carried out for this reason. As the main source of vitamin D is synthesis in the skin following sun exposure, several studies have investigated seasonal and geographical variation in cancer risk and survival [32-33]. One such study investigated the impact of season of diagnosis and residential region on the risk of death from lung cancer in Norwegian lung cancer patients [34]. Results suggested that vitamin D status at lung cancer diagnosis is of prognostic value and that cancer mortality decreases with increasing sun exposure[34]. Our results are in direct contrast to these studies, perhaps because of differences in the study cohorts. Firstly less than 20% of patients were deficient in vitamin D (Figure 2b). Secondly we showed that most tumours exhibited lower VDR expression than normal epithelial tissue. This means that the tumours would be less responsive to vitamin D, thus preventing its anti-tumour activities. Our immunohistochemistry results concur with a larger study on the expression of VDR in normal, premalignant and malignant bronchial tissue [6]. Furthermore, they are also consistent with genetic epidemiology work which shows that *VDR* polymorphisms which lead to less VDR function, are associated with malignancy in general [35]. This observation echoes smaller lung cancer studies which have shown that the *VDR* FokI polymorphism is associated with worse survival in NSCLC [36-37], whilst the TaqI polymorphism influences lung cancer risk, its effect being modified by age, gender and smoking habit [38]. It is also possible that unmeasured confounders, such as body weight, could have had an influence on our results.

Our study is limited to surgically resected cases, which led to relatively small numbers for the survival analyses; nevertheless the cohort remains competitive in the field for its size and

degree of characterization. The proportion of female patients is higher than the average, and many cases were quite advanced on pathological staging (stage IIIa or b) which may reduce the ability of the results to be generalized to other patient cohorts. We did not formally account for adjuvant therapy use in our analyses, since only 3 patients received it – given the low numbers we felt it would be uninformative to do so, but acknowledge that there is a small chance this could affect results. The study is also the first to report DBP immunohistochemistry in the lung. We were unable to confirm the location of DBP on macrophages by co-localisation of DBP and CD68 stains, due to a high level of background staining in the dually stained images [data not shown, available on request], although many of the slides show morphologically that the staining is on this cell type. We corrected our analyses for multiple tests, and acknowledge that it is only the unadjusted p value for DBP that reaches significance, since 4 quartiles were tested. However, given the marked difference in survival in this group, and the functional data we present to support our findings there remains potential for clinical significance.

In summary we have shown that low circulating DBP may predict poor prognosis in NSCLC, which we hypothesise is because of its role as a precursor to DBP-MAF. The results require independent replication and assessment in larger cohorts before we can be certain of the validity of DBP as a prognostic marker. If our results are validated by other groups further research to determine if DBP-MAF may be a useful therapy in the future could be warranted.

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Statement of contribution

AMT conceived the study, collected the tissue for immunohistochemistry, performed statistical analyses and drafted the manuscript. AM performed laboratory work and follow up data collection. LM, CW, GR, IT, YJ and MT performed laboratory work. SR and BN collected serum samples and baseline patient data. GL prepared all lung samples by inflation and analysed all immunohistochemistry. FB, MGT and BN also reviewed the manuscript, and BN supervised the work.

Conflict of interest statement

The authors have no real or perceived conflicts of interest to declare.

FIGURE LEGENDS

Figure 1: Pathological findings in the patients

The bar chart shows the pathological findings in the three patient groups. The majority of the lung cancer cases were either squamous or adenocarcinomas, with smaller numbers of small cell, large cell, bronchoalveolar cell and mixed cellularity tumours. The majority of the other intrathoracic cancers were oesophageal, whilst the bulk of the non-cancer cases were benign nodules.

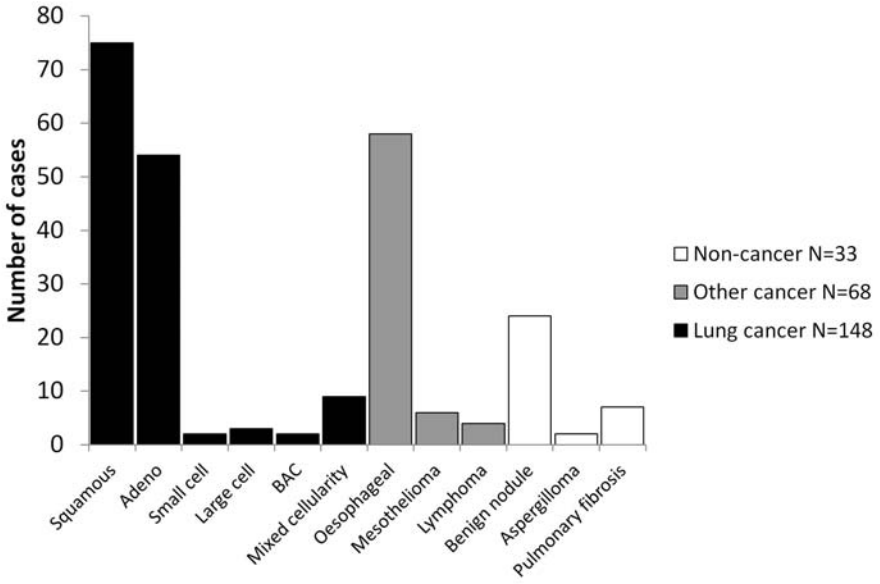

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Figure 2: Components of the vitamin D axis in cancer and non-cancer patients

(a) The bar chart shows mean (SEM) vitamin D and DBP levels in the 3 groups. Vitamin D did not differ between lung cancer and non-cancer patients ($p=0.06$), but was significantly lower in the other intra-thoracic malignancies ($p<0.01$). DBP was lower in

lung cancer than non-cancer patients (p=0.02) but did not differ from other cancers p=0.72).

(b) The pie chart shows clinical categories of vitamin D level in the lung cancer patients. The majority of lung cancer patients were sufficient in vitamin D.

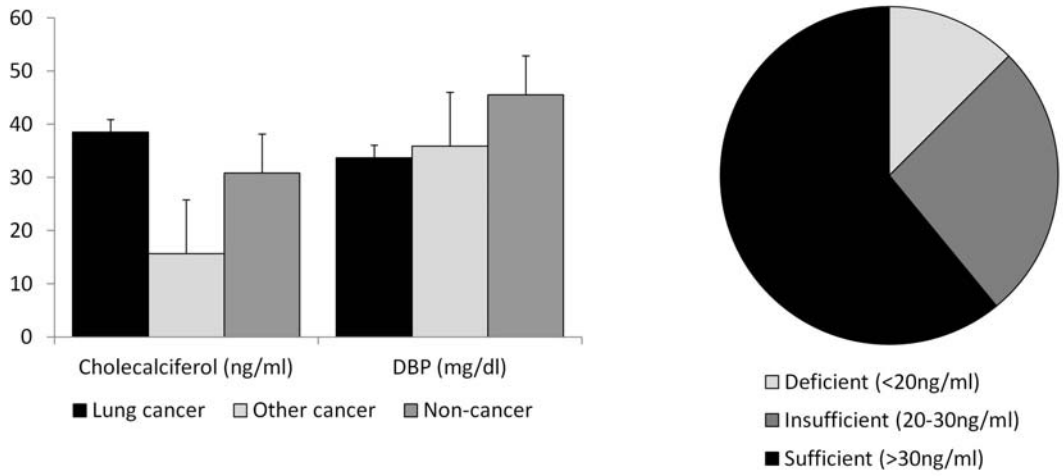

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Figure 3: Survival in the lung cancer patients

(a) Shows survival from the Cox regression analyses at the mean of all covariates, before the addition of DBP or vitamin D to the model.

(b) Shows survival at the mean of covariates, sub-stratified by DBP level. The top line (4.00) shows the highest quartile of DBP, whilst 3, 2 and 1 represent descending quartiles of DBP, with correspondingly lower survival.

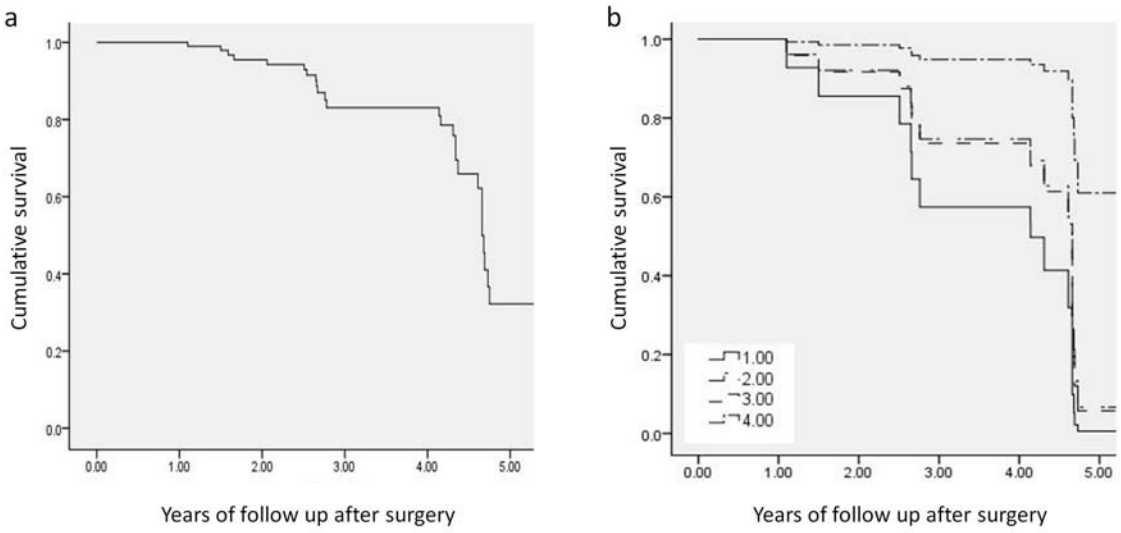

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Figure 4: VDR and DBP expression in lung tissue

(a) VDR stains strongly in bronchial epithelium, seen at 10x magnification (top) and more strongly than adjacent tumour tissue when seen at 40x (bottom).

(b) VDR generally exhibited less intense staining in tumour tissue; 2 tumours are shown at 20x and 40x (bottom) magnification.

(c) DBP is seen in blood (top) and airway secretions (bottom); 10x magnification.

(d) DBP is seen on macrophages (top) and at low intensity in an adenocarcinoma (bottom); both 40x magnification

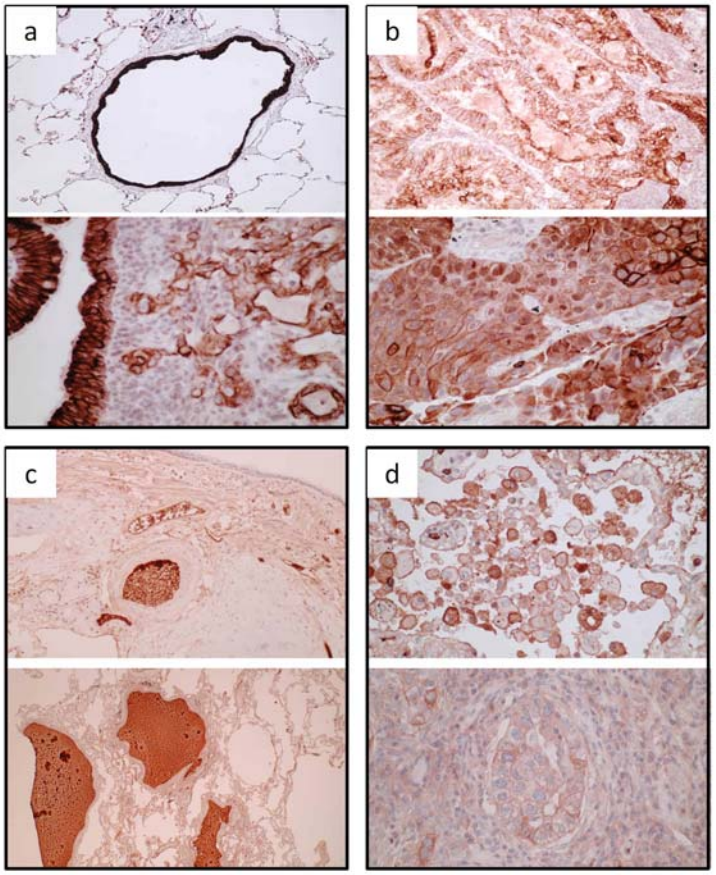

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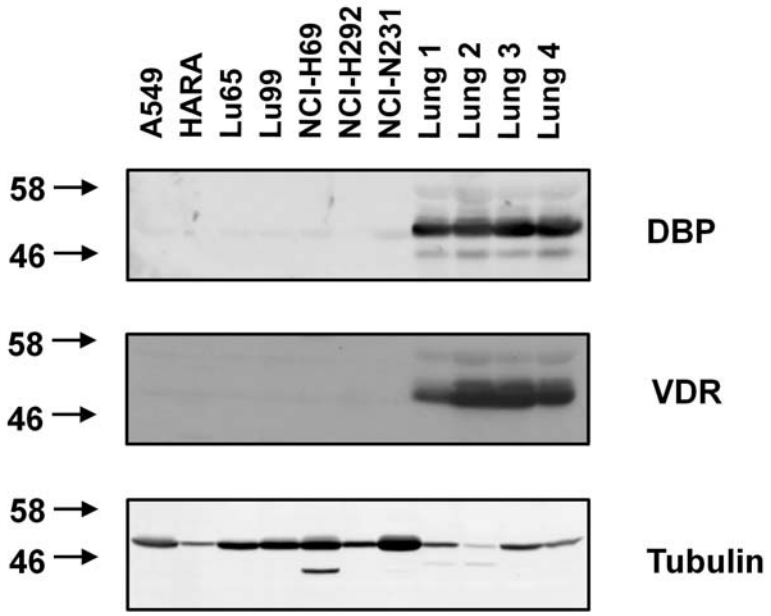
Figure 5: DBP and VDR expression by Western blotting in lung cancer cell lines and lung lysates

The blots show DBP, VDR and tubulin (control) expression in lung cancer cell lines (A549 to NCI-N231) and normal lung (lung 1-4). The scale on the left is in kDa. DBP and VDR were universally expressed by whole lung lysates. In the cancer cell lines DBP was expressed very weakly in some lines, and absent in most., whilst VDR was not expressed.



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