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Original Paper

Centrosome abnormalities are frequently observed in non-small-cell lung cancer and are associated with aneuploidy and cyclin E overexpression

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Abstract

Centrosome abnormalities are observed in human cancers and have been associated with aneuploidy, a driving force in tumour progression. However, the exact pathways that tend to cause centrosome abnormalities have not been fully elucidated in human tumours. Using a series of 68 non-small-cell lung carcinomas and an array of *in vitro* experiments, the relationship between centrosome abnormalities, aneuploidy, and the status of key G1 to S-phase transition cell-cycle molecules, involved in the regulation of centrosome duplication, was investigated. Centrosome amplification and structural abnormalities were common (53%), were strongly related to aneuploidy, and, surprisingly, were even seen in adjacent hyperplastic regions, suggesting the possibility that these are early lesions in lung carcinogenesis. Cyclin E and E2F1 overexpression, but not *p53* mutation, was observed to correlate with centrosome abnormalities *in vivo* ($p = 0.029$ and $p = 0.015$, respectively). This was further strengthened by the observation that cyclin E was specifically present in the nucleus and/or cytoplasm of the cells that contained centrosome aberrations. The cytoplasmic cyclin E signal may be attributed, in part, to the presence of truncated low-molecular-weight isoforms of cyclin E. In order to isolate the effect of cyclin E on the appearance of centrosome abnormalities, a U2OS tetracycline-repressible cyclin E cell line that has a normal centrosome profile by default was used. With this system, it was confirmed *in vitro* that persistent cyclin E overexpression is sufficient to cause the appearance of centrosome abnormalities.

Keywords: centrosome; aneuploidy; cyclin E; E2F1; non-small-cell lung cancer

Introduction

The presence of chromosomal instability (CIN) is one of the most common findings in human malignancies, including lung cancer. It consists of a variety of structural rearrangements at the genomic level (deletions, translocations, amplifications) as well as gains or losses of chromosomes. Aneuploidy may result from defects of the mitotic spindle checkpoint, defective cytokinesis or centrosome aberrations [1].

Centrosomes orchestrate normal mitosis by organizing the assembly of the bipolar spindle, forcing equal distribution of chromosomes to both daughter cells. The centrosome cycle is initiated at the G1 phase of the cell cycle, while duplication of centrosomes takes place through the S phase. At the end of the G2 phase, the two equal centrosomes are positioned at the opposite poles of the spindle [2,3].

A variety of tumours [4] display centrosome abnormalities, such as structural anomalies, excess pericentriolar material, impaired maturation, and disorientation of centrioles [2,3]. The presence of excess centrosomes, which are possibly non-functional, is the most common abnormality and may lead to multipolar spindle formation and asymmetric cell division. This event is believed to be a major cause for chromosome mis-segregation and aneuploidy. However, its underlying mechanisms have not yet been fully elucidated in lung carcinogenesis.

The centrosome duplication cycle is closely tied to DNA replication. Both are consequent on pRb phosphorylation and the resultant release of the E2F1 transcription factor. In particular, the activity of the E2F transcription factor is necessary for centrosome duplication [5]. One of the target genes of E2F1 is cyclin E, which is expressed during late G1 to early S phase and exhibits a peak expression level near the cell-cycle restriction point [6]. Whether E2F1 expression contributes to centrosome amplification in primary tumours, through its interaction with other cell-cycle regulators such as cyclin E, has not been determined. We have previously shown that E2F1 overexpression

is associated with aneuploidy [7,8] in non-small-cell lung cancer (NSCLC). Cyclin E overexpression has been related to adverse prognosis in lung cancer [9–14], but its mechanisms of action are not yet fully understood. Taking into account that cyclin E has been suggested to affect centrosome duplication [15–17] and that its aberrant expression may lead to CIN [6,18] via impairment of S-phase progression, it is important to investigate these associations in lung cancer.

Loss of p53 has a controversial effect on the centrosome profile. Some reports show that loss of p53, with concomitant overexpression of cyclin E, may synergistically induce centrosome amplification [19–21]. On the other hand, others have demonstrated that centrosome aberrations are not related to deregulated p53 function [4,22,23]. None of the above correlations has been examined in NSCLC.

Based on the above, we set out to examine the frequency of centrosome abnormalities in lung cancer and their putative association with aneuploidy and deregulated expression of key cell-cycle modulators implicated in the centrosome cycle. As far as we are aware, such a study has not been reported for non-small-cell lung cancer.

Materials and methods

Tumour specimens

Formalin-fixed, paraffin-embedded biopsy material from a total of 68 NSCLCs and corresponding normal lung tissue was analysed (Table 1). Tumours were classified according to World Health Organization criteria and the staging system. Patients had received no chemotherapy, radiotherapy, or immunotherapy before surgery. Due to the recent collection of the specimens, survival analysis was not feasible. Collection, coding, and analysis of the tissues and data banks were performed in accordance with the revised (1983) Helsinki Declaration of 1975 for ethical standards. The study was approved by the Ethics Committee of the University of Athens.

Cyclin E-inducible cellular system

U2OS-derived cells with tetracycline-repressible expression of wild-type full-length human cyclin E were generated as previously described [24]. The cells were grown in DMEM medium supplemented with 10% fetal calf serum and antibiotics in a 5% CO_2 atmosphere [24].

Immunohistochemistry (IHC)–indirect immunofluoresence (IF)

Antibodies

For IHC, the following antibodies were used: anti-γ-tubulin (GTU-88; class: IgG_1 mouse monoclonal; epitope: residues 38–53; Sigma, Anti-Sel, Greece); anti-cyclin E (13A3; class: IgG_{2a} mouse monoclonal; epitope: not determined; Abcam, Anti-Sel, Greece); anti-E2F1 (KH95; class: IgG_{2a} mouse monoclonal; epitope: Rb binding domain of E2F-1 p60; Santa-Cruz, Bioanalytica, Greece); anti-p53 (DO7; class: IgG_{2b} mouse monoclonal; epitope: residues 1–45; DAKO, Kalifronas, Greece).

For IF, the following antibodies were used: anti-cyclin E (C-19; class: IgG rabbit polyclonal; epitope: C-terminus; Santa-Cruz, Anti-Sel, Greece); anti-γ-tubulin (GTU-88; class: IgG_1 mouse monoclonal; epitope: residues 38–53; Sigma, Anti-Sel, Greece).

Table 1. Summary of the clinicopathological features and p53, cyclin E, E2F1, ploidy/degree of hyperploidy and centrosome amplification status in our NSCLCs series

Patient characteristics	
Age	
Median (IQR)	67.5 (7.0)
Sex	
Male	65
Female	3
Smoking habits	
Smokers	58
Non-smokers	4
Tumour characteristics	
Histology	
Adenocarcinoma	19
Squamous	40
Stage	
I	35
II	22
III	8
Differentiation	
Low	27
Medium/high	28
p53 IHC status	
Wild type	17
Mutated	49
Cyclin E IHC levels	
Median (IQR)	21.5 (18.6)
E2F1 IHC levels	
Median (IQR)	28.3 (20.4)
Ploidy	
Diploid	10
Aneuploid	33
Degree of hyperploidy	
Median (IQR)	10.3 (16.6)
Centrosome amplification	
Absent	32
Present	36

Method

IHC and IF were performed according to a previously published protocol [7].

Controls

The negative controls for cyclin E were sections incubated only with pre-immune serum instead of primary antibodies. Normal bronchial epithelium (Figure 1), lung stroma, and alveoli (γ-tubulin IF staining) were

used as internal controls for the number and morphology of centrosomes [4]. Controls for E2F1 and p53 staining have been previously reported [8,25].

Evaluation

Scoring of E2F1 and p53 IHC results was performed as previously described [8,25]. Cyclin E labelling index (LI) was assessed as the percentage of stained tumour nuclei in IHC [9–14]. In IF, cells were considered positive for cyclin E expression when the nucleus and/or cytoplasm were distinctly stained. Representative positively stained samples were also confirmed by western blot analysis of cytoplasmic and nuclear extracts from the respective tissues.

To determine centrosome aberrations in each sample by IF, we examined six microscopic fields. Centrosome abnormalities were graded according to the percentage of cells having more than two centrosomes and the presence of structural irregularities (greater diameter and elongated shape in comparison with centrosomes of normal lung stroma, alveoli, and bronchi): negative (<5%); low (5–10%); medium (10–30%); high (>30%) (criteria based on refs 4, 23, and 26). IHC and IF slides were examined by four independent observers (MK, KE, KA, and VG).

Protein extraction, cell fractionation, and western blot analysis

Protein extracts

Total protein and nuclear and cytoplasmic fractions were obtained from matched frozen normal and tumour tissues as previously described [7,27].

Antibodies and controls

For western blot analysis, the following antibodies were used: anti-cyclin E (HE12; class: IgG_{2b} mouse monoclonal; epitope: COOH terminus [28]; Santa Cruz, Bioanalytica, Greece). Extracts from MCF-7 cancer breast cell line were used as a positive control for cyclin E's isoform analysis [29]. Anti-actin (AC-15; class: IgG_1 mouse monoclonal; Abcam, AntiSel, Athens, Greece) and anti-lamin B2 (LN-43; class: IgG_1 mouse monoclonal; Abcam, AntiSel, Athens, Greece) were used to assess equal loading of total protein per sample and specificity of the signal from the nuclear–cytoplasmic extracts, respectively.

Gel electrophoresis and blotting

SDS-PAGE analysis and evaluation of results have been previously reported [27].

Signal development and quantitation

Blots were blocked for 3 h in 2.5% gelatin/TBS-T (TBS-T: TBS, 0.1% Tween-20) at room temperature. Subsequently, membranes were incubated overnight with the primary antibody (1/250 dilution) at 4 °C, followed by 1 h incubation with the appropriate secondary antibody (1/5000 dilution) at room temperature. Signal development and quantitation were performed following a previously published protocol [27].

Ploidy analysis

Ploidy analysis was performed as previously described [30], with certain modifications in the preparation of tissue material. The examined tumour cells were collected from three 50 μm sections after pepsin treatment and prepared as cytological material. The degree of hyperploidy (DH), a surrogate marker of ploidy indicating the percentage of cells with a DNA content greater than 5c, was also evaluated [30].

Statistical analysis

Data analysis was performed with the R language for statistics [31]. Non-parametric tests (Spearman and Kendall rank correlation, Wilcoxon and Kruskal rank sum tests) were used when necessary and adjustment for multiple comparisons was made where applicable. Results were considered significant when $p < 0.05$.

Results

Centrosome abnormalities are common in NSCLCs

More than half of our specimens (53%) harboured cells with three or more centrosomes (centrosome amplification), the presence of which was almost always accompanied by centrosomal structural irregularities (chi-square $p < 0.001$). There was no significant difference between the degree of centrosome abnormality (low, medium, high) and the status of cyclin E, E2F1, and p53. Thus, we sub-grouped the cases into positive and negative depending on the presence or absence of centrosome abnormalities. Centrosome abnormalities were not associated with clinicopathological markers such as stage, tumour grade, and histological subtype (Table 2).

We noticed that tumour cells had larger and elongated centrosomes (Figures 1 and 2) than cells of the normal epithelium (Figure 1). In certain cases, some intriguing centrosomal arrangement patterns were observed. In particular, multiple centrosomes would gather at one side of the nucleus, like a comet tail, or form a 'beaded necklace' around it (Figure 2). Tumour cells with centrosome abnormalities were often grouped into distinct patches in a microscopic field and surrounded by tumour cells with phenotypically normal centrosomes.

Thirteen specimens included regions of adjacent hyperplastic epithelium. Ten of these contained cells with normal centrosomes, while three specimens had centrosome abnormalities. In these cells, the centrosomes were larger but retained their normal orientation towards the bronchial lumen (Figure 1).

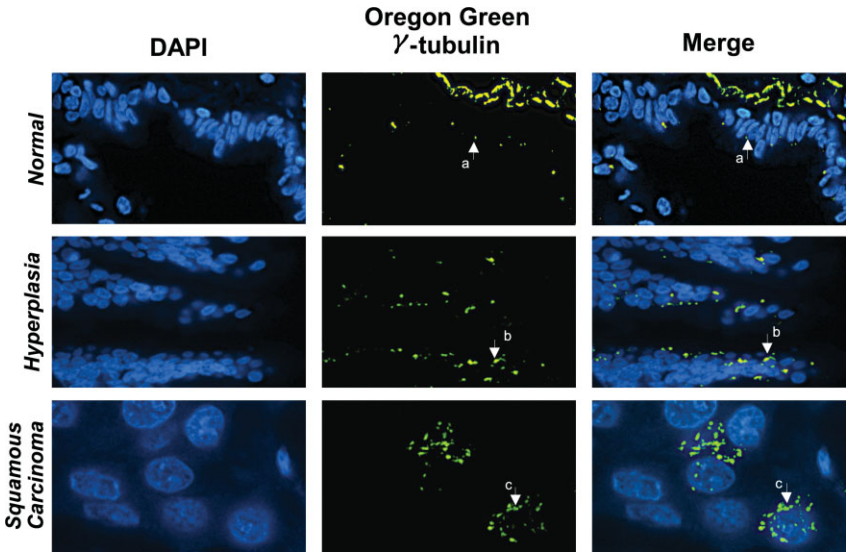

Figure 1. Immunofluorescent detection of centrosomes in normal, hyperplastic, and malignant lung tissue. Specimens were stained with γ-tubulin (Oregon Green), while the nuclei were counter-stained with DAPI. Normal bronchial epithelium, original magnification $\times$ 400 (case 56). Hyperplastic bronchial epithelium, original magnification $\times$ 400 (case 56). Squamous lung carcinoma (case 51), original magnification $\times$ 1000. The arrows respectively indicate (a) a normal centrosome, (b) centrosome abnormalities in hyperplasia, and (c) more intense centrosome amplification in a squamous lung carcinoma

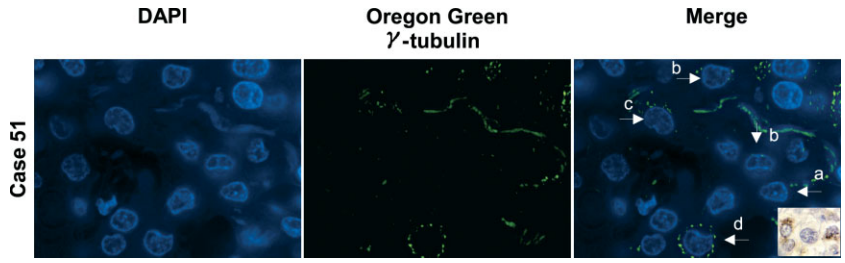

Figure 2. Types of centrosome aberration in non-small-cell lung cancer. NSCLCs present a variety of centrosome abnormalities. Case 51 (squamous lung carcinoma) was stained with γ-tubulin (Oregon Green), while the nuclei were counter-stained with DAPI. Arrows and letters depict the cells with corresponding centrosome abnormalities: (a) tumour cell that has one normal-looking centrosome; (b) cells with more than two centrosomes; (c) multiple centrosomes gathered on one side of the nucleus forming a 'comet tail'; and (d) multiple centrosomes positioned around the nucleus resembling a 'beaded necklace'. IHC of the same specimen, incubated with γ-tubulin (inset) shows nuclei with centrosome amplification; original magnification $\times$ 640

Centrosome abnormalities are associated with aneuploidy in NSCLCs

We determined the ploidy status (Table 1) in 43 tumours, 33 of which were aneuploid (77%). We also recorded the degree of hyperploidy, whose median value was 10.3%. The majority of aneuploid tumours had centrosome abnormalities, while the majority of diploid tumours had normal centrosomes (Table 2) and this association was significant ($p = 0.002$). In addition, the degree of hyperploidy was higher in tumours with centrosome abnormalities ($p = 0.006$) and in tumours with low (poor) differentiation ($p = 0.006$).

Centrosome abnormalities are associated with cyclin E overexpression

To examine the link between cell cycle deregulation and centrosome abnormalities, we proceeded with the immunohistochemical evaluation of cyclin E (Figure 3A), E2F1, and p53 (results in Tables 1 and 2). Staining for cyclin E was predominantly in the nucleus of tumour cells. The adjacent normal epithelium was negative for cyclin E staining. As expected [8,32], E2F1 overexpression was positively associated with high cyclin E expression ($p = 0.029$) and an increased degree of hyperploidy ($p = 0.040$). In a simple linear model (adjusted $r^2 = 0.14$, $p = 0.010$), E2F1 expression was highest when centrosome abnormalities were present ($p = 0.015$) and differentiation was low ($p = 0.027$).

The above results prompted us to investigate the relationship between cyclin E, cellular proliferation, and centrosome abnormalities further. Indeed, specimens with high cyclin E expression had increased proliferation (correlation with Ki67, data from ref 8, $p = 0.047$) and frequently harboured centrosome abnormalities ($p = 0.029$), as shown by a generalized linear model. To visualize this interaction, we performed double immunofluorescence to examine the localization of aberrant centrosomes and cyclin E in tumour cells. In the majority of specimens (88%), cells

Table 2. Correlations among centrosome abnormalities, cyclin E, E2F1 and p53 status and their associations with clinicopathological features and ploidy/degree of hyperploidy status in our NSCLCs series

	Centrosome abnormalities			Cyclin E		E2F1		p53 IHC		
	Absent	Present	*p* value	Mean (SE)	*p* value	Mean (SE)	*p* value	Wild-type	Mutated	*p* value
Tumour characteristics										
Histology										
Adenocarcinoma	9	10		17.8 (2.91)		31.7 (3.35)		4	13	
Squamous carcinoma	17	23	0.94	23.9 (1.91)	0.14	33.9 (2.61)	0.81	11	29	0.98
Stage										
I	18	17		19.5 (1.99)		31.8 (3.22)		9	25	
II	9	13		24.8 (2.95)		31.2 (3.11)		4	17	
III	4	4	0.73	23.2 (4.70)	0.06*	36.5 (3.83)	0.21*	3	5	0.58
Differentiation										
Low	14	13		23.8 (2.50)		37.0 (2.85)		7	20	
Medium/high	11	17	0.50	21.0 (2.08)	0.60	28.2 (2.45)	0.07	7	20	0.76
Cyclin E IHC levels										
Mean values (SE)	19.4 (2.32)	22.8 (2.42)	0.03†	—		rho = 0.27	0.03	18.9 (3.27)	22.1 (1.98)	0.39
E2F1 IHC levels										
Mean values (SE)	30.0 (3.23)	34.0 (2.83)	0.28	rho = 0.27	0.03	—		31.1 (4.16)	31.8 (2.45)	0.85
p53 IHC status										
Wild-type	9	8		18.9 (3.27)		31.1 (4.16)		—	—	
Mutated	22	27	0.77	22.1 (1.98)	0.39	31.8 (2.45)	0.85	—	—	
Ploidy										
Aneuploid	10	23		21.8 (1.97)		32.9 (1.80)		8	25	
Diploid	9	1	0.002	22.8 (2.02)	0.99	24.9 (3.29)	0.06	5	5	0.14
Degree of hyperploidy										
Mean values (SE)	9.0 (2.23)	18.9 (3.08)	0.005	rho = −0.11	0.50	tau = 0.23	0.04	13.7 (3.29)	14.9 (2.50)	0.99
Centrosome abnormalities										
Absent	—	—		19.4 (2.32)		30.0 (3.23)		9	22	
Present	—	—		22.8 (2.42)	0.03†	34.0 (2.83)	0.28	8	27	0.77

* ANOVA.
† Generalized linear model.
rho: Spearman's rho; tau: Kendall's tau; mean (SE): mean value (standard error).

with centrosome abnormalities also showed a strong signal for nuclear or cytoplasmic cyclin E, or both. Only a few specimens (12%) contained cells with centrosome abnormalities but without apparent cyclin E expression. The cytoplasmic signal of cyclin E was not diffuse, but visible as dots that tended to cover centrosomes (Figure 4). The expression of cyclin E was also quantified by western blotting of total, nuclear, and cytoplasmic protein extracts of specimens with centrosome abnormalities. Interestingly, low-molecular-weight (LMW) isoforms of cyclin E were observed as five bands in both nuclear and cytoplasmic extracts from tumours. The concentration of full-length cyclin E (50 kD) was approximately equal between normal and tumour samples, but the LMW isoforms were overexpressed in tumours (Figure 3B).

The *in vivo* findings are recapitulated *in vitro* in a cyclin E-inducible system

We used U2OS-derived cells with tetracycline-repressible expression of cyclin E to recapitulate our *in vivo* findings. This inducible system expresses wild-type p53 and pRb and has a normal centrosome profile by default [24,33]. The use of this system isolates the influence of cyclin E on centrosomes and minimizes the possibility of a confounding effect of the p53 and pRb molecules, which are known to affect the centrosome profile [3]. Indeed, U2OS cells treated with tetracycline only carried one or two centrosomes per cell and showed moderate intrinsic cyclin E expression (20% of the cells) with light IF staining (Figure 5).

After 2 days without tetracycline, IF staining for cyclin E was considerably more intense but the centrosome profile was still normal. At the fourth day without tetracycline, the majority (70%) of cells that were positive for cyclin E carried various centrosome abnormalities (Figure 5). Gradually, the number of cells overexpressing cyclin E increased and the presence of centrosome abnormalities was more pronounced. At the eighth day, many cells had more than ten centrosomes, one or two of which seemed to stand out, while the rest gathered at the one side of the nucleus, forming a 'comet tail'. No mitoses were observed. It should be noted that some nuclei had adopted a senescence-like phenotype [34] and regions of heterochromatin began to appear (Figure 5). Heterochromatin formation was particularly evident after 10 days without tetracycline.

Discussion

For the first time in primary NSCLCs, we provide evidence that centrosome aberrations are very common and are strongly related to aneuploidy. Centrosome

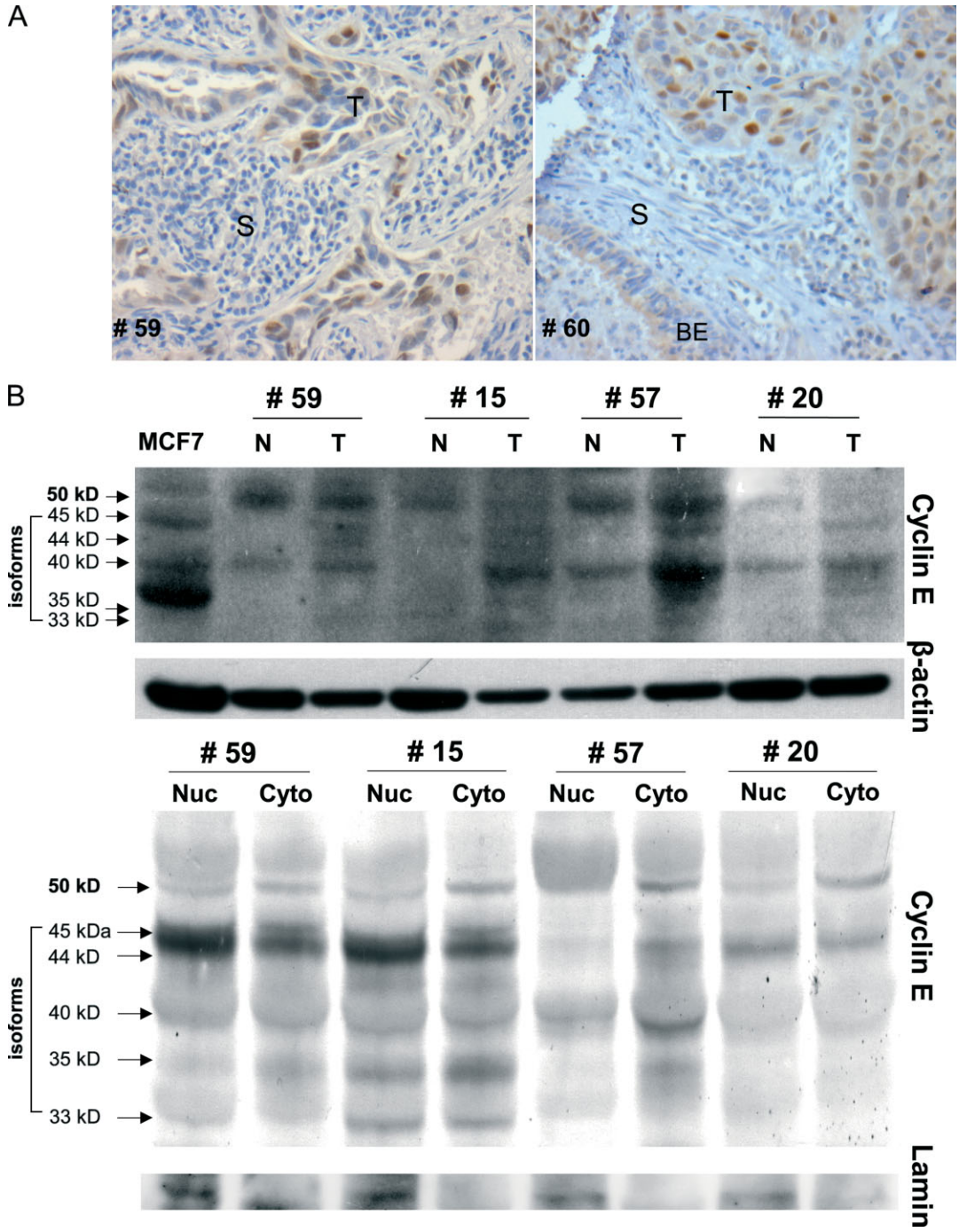

Figure 3. (A). Immunohistochemical expression of cyclin E in NSCLCs. Representative cases of adenocarcinoma (#59) and squamous cell carcinoma (#60) showing overexpression of cyclin E, original magnification × 200. Compared with tumour tissue (T), bronchial epithelium (BE) and lung stroma (S) do not express cyclin E. (B) Western blot analysis of cyclin E in NSCLCs. Expression analysis of cyclin E in representative cases 59 (adenocarcinoma), 15 (squamous cell carcinoma), 57 (adenocarcinoma) and 20 (squamous cell carcinoma) and their normal counterparts. The arrows indicate the full-length cyclin E (50 kD) and its isoforms (45, 44, 40, 35, and 33 kD). The expression of full-length cyclin E is approximately equal in normal and tumour samples, while its isoforms are overexpressed in tumour specimens. MCF7 extracts were used as a control for cyclin E isoforms. Equal protein loading for both counterparts was estimated by the presence of actin. Expression analysis of nuclear and cytoplasmic fractions of the above cases shows that cyclin E isoforms are present in both fractions. The specificity of the signal was assessed by anti-lamin B2

abnormalities may result from improper centrosome maturation and centrosome overduplication due to S-phase prolongation or aborted mitosis [3]. Furthermore, numerical and structural aberrations of centrosomes almost always co-existed. We observed that tumour cells with centrosome abnormalities usually displayed a local distribution and gathered into patches in the tumour nests of NSCLCs, possibly the result of clonal expansion of deregulated cells.

According to our findings, cell populations with centrosome abnormalities are frequently hyperploid. A minority of tumours with centrosome aberrations

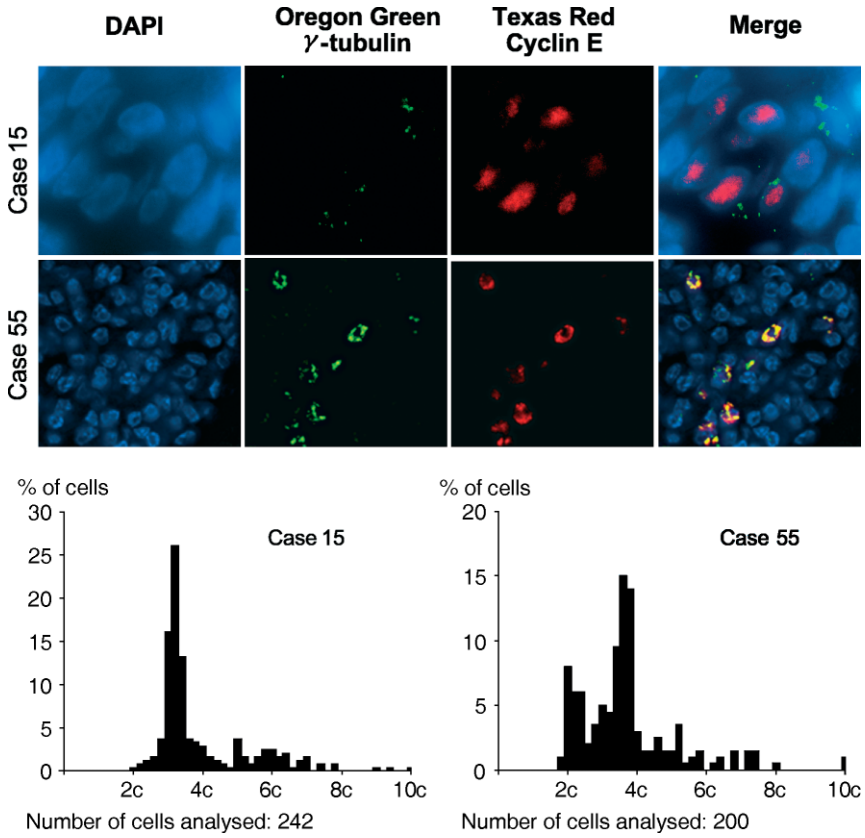

Figure 4. Centrosome amplification in NSCLC tumour cells expressing cyclin E. Specimens were double-stained with γ-tubulin (Oregon Green) and cyclin E (Texas Red) antibodies, while the nuclei were counter-stained with DAPI. Merge shows combined images of γ-tubulin, cyclin E, and DAPI staining. Case 15, squamous cell carcinoma, original magnification $\times$ 1000: a group of nuclei stain for cyclin E. Some of them have excess centrosomes. In the same image, there is a nucleus negative for cyclin E that also has an abnormal number of centrosomes. Case 55, squamous cell carcinoma, original magnification $\times$ 400: cyclin E staining is cytoplasmic and coincides with γ-tubulin staining. Ploidy analysis demonstrated aneuploidy in both cases (lower panel)

managed to compensate and maintain a diploid genome, a finding that has also been observed in breast cancer [22]. It is possible that this is only a transient state that will finally terminate in aneuploidy. On the other hand, judging from the presence of aneuploid tumours without centrosome aberrations in our study, other molecular mechanisms are also capable of leading to aneuploidy [1].

Of particular interest is the detection of centrosome aberrations in adjacent hyperplastic regions. Even though this phenomenon was not universal, it was common enough (23% of samples with hyperplastic regions) to indicate the possibility that deregulation of the centrosome cycle and function is an early step in tumour progression. This hypothesis is in line with other studies that show the early appearance of centrosome aberrations in cancer progression [22,23,35–37]. We also have to acknowledge the possibility that according to the theory of field cancerization [38,39], these nearby cells might belong to a 'genetically altered field' that suffers from various molecular defects, centrosomal in this case, without other striking phenotypic abnormalities. Furthermore, persistent inflammation, which enhances hyperplasia in the smoker's bronchi, can also induce centrosome aberrations [40]. Whatever the exact explanation may be, a specific investigation of centrosomal status and function in pure hyperplasia is warranted to determine the subsequent progression of these lesions.

The centrosome duplication cycle is definitely parallel to DNA replication [3]. These simultaneous procedures are connected via pRb phosphorylation and the subsequent release of E2F1 transcription factor. The cyclin E/cdk2 complex is also able to phosphorylate pRb [6] and further enhance E2F1 release in a feedback loop mechanism.

Centrosome duplication requires E2F transcription factor activity [5] and cyclin A/cdk2 and might depend particularly on E2F3 inactivation [41]. Centrosome amplification and low differentiation were associated with increased E2F1 levels in our study, a novel finding that has not been previously reported. In addition, the relationship between degree of hyperploidy and E2F1 overexpression agrees with our previous studies [7,8] and implies yet another mechanism through which E2F1 may render the cells liable to mitotic defects and genomic instability. The putative mediators of E2F1 action could include several downstream effectors, such as cyclin E [42]. In particular, the E2F1–cyclin E axis may induce centrosome amplification that can lead to aneuploidy in NSCLCs (Figure 6), although other pathways may also be involved.

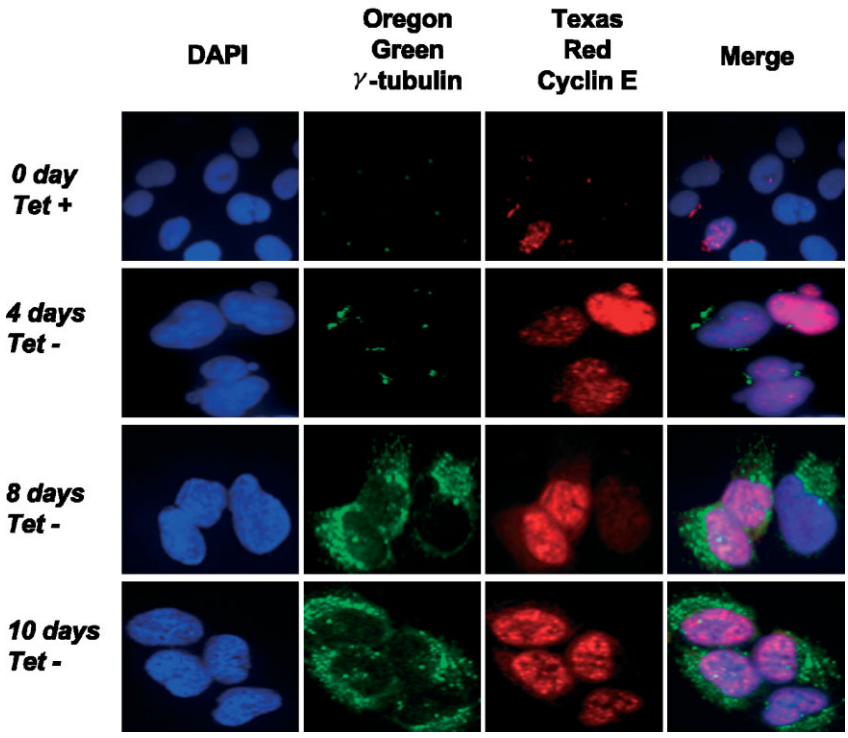

Figure 5. The impact of ectopic cyclin E expression on centrosome duplication in U2OS cells. Cells were double-stained with γ-tubulin (Oregon Green) and cyclin E (Texas Red) antibodies, while the nuclei were counter-stained with DAPI. Merge shows combined signals of γ-tubulin, cyclin E, and DAPI staining. Control U2OS cells, treated with tetracycline (tet +) harbour one or two centrosomes per cell. Cells were then deprived of tetracycline (tet −) and cyclin E overexpression led to centrosome amplification. The nuclei on 8 and 10 days contain regions of heterochromatin. Images at day 0, original magnification × 640; other images, original magnification × 1000

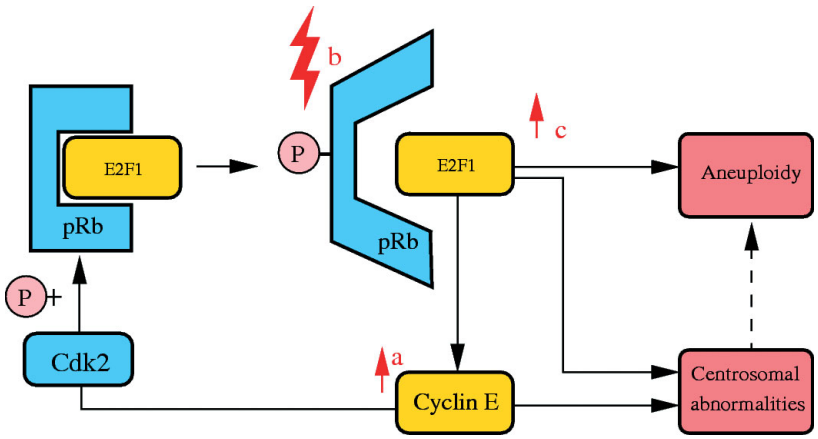

Figure 6. A model showing the association of the E2F1–cyclin E axis with centrosome abnormalities and aneuploidy. In normal cells, cyclin E promotes the G1-to-S transition by forming a complex with cdk2 that is able to phosphorylate pRb (in addition to the cyclin D1/cdk4 and cdk6 complexes). This phosphorylation enables the release and activation of E2F1, which binds to its transcriptional target genes, including cyclin E, and creates a positive feedback loop. In tumour cells, the above pathway may be impaired when *RB1* is mutated (b) or cyclin E (a) and/or E2F1 (c) are overexpressed. Deregulation of the E2F1–cyclin E axis may cause S-phase prolongation and subsequently induce centrosome abnormalities. On the other hand, E2F1 could also lead to centrosome abnormalities through other mechanisms. Our study shows that centrosome abnormalities are associated with aneuploidy in NSCLCs

Cyclin E is highly expressed in specimens with centrosome abnormalities and is also specifically present in the nucleus and/or cytoplasm of the cells that contained centrosome aberrations (Figure 4). Almost 90% of samples with centrosome abnormalities displayed this pattern. Supportive evidence shows that the cyclin E/cdk2 complex is required for centrosome duplication in *Xenopus* egg extracts [15] and controls the expression of several proteins responsible for centrosome stabilization (nucleophosmin/B23) and duplication (Mps1) [43–45]. The presence of cytoplasmic cyclin E may be attributed to the ability of the cyclin E/cdk2 complex to shuttle between the nucleus and the cytoplasm [46].

This is the first time that cyclin E's LMW isoforms, extensively studied in breast cancer (ref 28 and refs cited therein) [29], have been found in NSCLCs, in both nuclear and cytoplasmic extracts. These isoforms are the result of post-translational modification at the $-NH_2$ end [28,47,48] and have been observed in breast cancer with the same antibody. It is possible that what is perceived as cyclin E overexpression by *in situ* techniques may in fact sometimes represent up-regulation of LMW isoforms, as shown in our western blot analysis. The exact significance of the accumulation of cyclin E on the centrosomes and the role of LMW isoforms in centrosome function and phenotype are currently unclear. Whether they induce aneuploidy and CIN, as shown in breast and colon cancer [28,29,49], and confer an adverse prognosis in NSCLC should be further investigated.

The *in vitro* interventional experiments indicate that persistent cyclin E overexpression is sufficient to cause the appearance of centrosome abnormalities (Figure 5). Based on our *in vivo* results, cyclin E overexpression can lead to S-phase entry, aneuploidy and genomic instability over time. Indeed, using the same cell line, we have shown that cyclin E overexpression leads to the accumulation of cells in S and G2 phases and cells with increased DNA content appear progressively [24]. This is consistent with the possibility that cyclin E might prolong the S phase and dissociate the cell and centrosome cycles. Interestingly, after 8 days, the appearance of a 'comet-tail' formation of centrosomes was seen in parallel with changes in nuclear morphology and chromatin structure similar to those that have been described in senescent cells [35].

It has been suggested that loss of p53 and overexpression of cyclin E synergistically induce centrosome amplification [19–21]. However, our data do not support this conclusion in primary NSCLC. Loss of p53 has been reported to promote the accumulation of functional centrosomes in mouse embryo fibroblasts [50]. However, in accordance with other studies in a variety of malignancies [4,22,23], we did not observe this association.

The role of centrosome abnormalities in cancer has been a field of active research. It is now evident that centrosome abnormalities reflect the status of important molecular pathways, such as the E2F1–cyclin E axis, and are strongly associated with the appearance of aneuploidy. Most importantly, centrosome abnormalities have been found in very early lesions, including hyperplasia in our study. This information should provide a stepping stone for further research that will evaluate centrosome abnormalities as putative prognostic markers or therapeutic targets.

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