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Cyclin E dysregulation and chromosomal instability in endometrial cancer

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Deregulation of cyclin E, an activator of cyclin-dependent kinase 2 (Cdk2), has been associated with a broad spectrum of human malignancies. Yet the mechanism linking abnormal cyclin E expression to carcinogenesis is largely unknown. The gene encoding the F-box protein hCdc4, a key component of the molecular machinery that targets cyclin E for degradation, is frequently mutated in endometrial cancer, leading to deregulation of cyclin E expression. Here we show that *hCDC4* gene mutation and hyperphosphorylation of cyclin E, a parameter that usually correlates with *hCDC4* mutation, have a strong statistically significant association with polypoidy and aneuploidy in endometrial cancer. On the contrary, elevated expression of cyclin E by itself was not significantly correlated with polyploidy or aneuploidy when tumors of similar grade are evaluated. These data suggest that impairment of cell cycle regulated proteolysis of cyclin E may be linked to carcinogenesis by promoting genomic instability.
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Cyclin E, an activator of cyclin-dependent kinase 2 (Cdk2), is a critical regulator of the G_1–S phase transition in mammalian cells. Although the substrates of cyclin E/Cdk2 are not well defined, the kinase complex has been shown to regulate a number of important S phase processes such as pRb phosphorylation, histone biosynthesis, and pre-replication complex assembly (Reed, 1996; Ekholm and Reed, 2000).

In normal cells, cyclin E protein levels are periodic, peaking at the G_1/S phase boundary and declining once an S phase program is initiated. The decline of cyclin E protein level occurs through a combination of down-regulation of cyclin E transcription and ubiquitin-mediated proteolysis of cyclin E protein (Clurman *et al*., 1996; Won and Reed, 1996; Reed, 1997). Proteolysis is initiated by auto-phosphorylation of either residue Thr62 or Thr380 by its catalytic partner Cdk2 (Strohmaier *et al*., 2001). Phosphorylated cyclin E is then targeted for ubiquitylation by a ligase belonging to the SCF family. SCF ligases are composed of an invariable core of Skp1, Cul1 (Cdc53), and Roc1, bound to one of several F-box proteins that provide substrate specificity. Recently, the F-box protein that targets phosphorylated cyclin E for ubiquitylation was identified as hCdc4, also designated as Fbw7 or Archipeligo (Koepp *et al*., 2001; Moberg *et al*., 2001; Strohmaier *et al*., 2001).

Abnormalities involving cyclin E have been observed in many types of human tumors. These abnormalities include elevated protein levels (Donnellan and Chetty, 1999), deregulation relative to cell cycle progression (Keyomarsi *et al*., 1995; Erlanson and Landberg, 1998), and the presence of low-molecular-weight forms of cyclin E (Keyomarsi *et al*., 2002). Numerous studies have linked these aberrant phenotypes to advanced disease, and in breast cancer cyclin E abnormalities represent one of the strongest markers of poor patient prognosis (Keyomarsi *et al*., 2002). The oncogenic potential of dysregulated cyclin E has also been demonstrated experimentally. Ectopic expression of cyclin E can induce premature onset of DNA synthesis in cultured cells (Resnitzky *et al*., 1994). Furthermore, deregulated expression of cyclin E in the mammary epithelium of mice has been shown to induce the formation of tumors (Bortner and Rosenberg, 1997). Aberrant cyclin E expression has also been shown to induce chromosome instability, increasing the frequency of both aneuploid and polyploid cells (Spruck *et al*., 1999). Conversely, low cyclin E levels have been correlated with fewer chromosomal imbalances in breast carcinomas (Blegen *et al*., 2003).

The molecular mechanism(s) underlying the association between cyclin E dysregulation and chromosome instability, a characteristic that is strongly associated

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with human tumorigenesis, is not understood. Also, the proposed role deregulated cyclin E plays in generating chromosome instability in tumors has never been evaluated. In this study, we perform a comprehensive cytogenetic analysis of primary endometrial adenocarcinomas to shed light on how cyclin E abnormalities contribute to chromosome instability during human tumorigenesis.

The tumors used in our study had been previously analysed for cyclin E protein level and phosphorylation status by Western blot analysis and screened for mutations in the *hCDC4* gene by single-strand conformation polymorphism (SSCP) analysis (Spruck *et al*., 2002). A summary of these data is shown in Table 1. Fluorescent *in situ* hybridization (FISH) analysis was subsequently performed to determine the degree of genomic instability in each tumor specimen. FISH probes were chosen based on published comparative genomic hybridization (CGH) data on endometrial tumors (Suzuki *et al*., 1997). Probes used included those specific for chromosomes 1q and 8q, which show frequent alterations in endometrial tumors, and chromosome 11, which is altered infrequently. Additionally, probes specific for c-Myc (chromosome 8) and IGH (chromosome 14) were included in our analysis. All five probes were pre-screened using 20 benign endometrial tissue samples to determine the background frequency of nonspecific hybridizations. Samples of representative FISH analyses are shown in Figure 1. The FISH data for all tumors analysed are summarized in Table 1. Alterations were found at three or more loci in 15 of 28 tumors analysed. Furthermore, alterations at all five loci examined were observed in five tumors. In four of these cases, four hybridization signals were observed for each marker, strongly suggesting a tetraploid DNA content. Conversely, six tumors demonstrated two hybridization signals at all five loci, suggesting a diploid DNA content.

Cytometric analysis was performed on isolated nuclei from 17 tumors to complement and confirm the FISH analysis. In all, 100 nuclei were analysed from each tumor specimen and the results were plotted as DNA histograms (Figure 1). A total of 13 tumors were found to contain an aberrant (nondiploid) DNA content (Table 1). These results confirmed the conclusions of FISH analysis in 11 tumors that displayed abnormalities based on at least three different probes. The histograms of four tumors demonstrated a single 4C peak, suggesting a tetraploid DNA content. Additionally, three tumors displayed a heterogeneous DNA content displaying peaks at both 4C and an aneuploid peak (see legend, Figure 1). Of the four tumors that were characterized as tetraploid by FISH, all showed either a single 4C peak or were characterized as tetraploid/aneuploid by cytometry. Additionally, aneuploid DNA histograms were observed for six tumors. Discordant results were observed in only two cases where tumors displayed an aneuploid DNA histogram, but did not demonstrate abnormalities of more than two markers by FISH. These discrepancies can be rationalized by assuming that the aneuploidy did not include the few chromosomes selected for FISH analysis.

We next determined whether statistically significant associations exist in these tumors between the status of cyclin E and *hCDC4* and chromosome abnormalities, as measured by the number of altered loci determined by FISH. Statistical analyses were performed using the Mann–Whitney *U* test. Cyclin E levels (low *vs* intermediate/high) showed only borderline significance towards more affected loci in tumors with intermediate/high levels of cyclin E ($P = 0.072$). However, the presence of *hCDC4* mutations or phosphorylated cyclin E protein were found to be statistically significantly associated with severe chromosomal abnormalities ($P = 0.021$ for *hCDC4* mutations; $P = 0.00039$ for phosphorylated cyclin E).

Although deregulated cyclin E has been shown to induce chromosome instability in cultured cells, this association had never been demonstrated in primary tumors. In this study, we demonstrate in endometrial tumors that *hCDC4* gene mutations or phosphorylated cyclin E are significantly associated with a chromosome instability phenotype. These results suggest that impairment of the proteolytic processes that mediate cyclin E degradation may have profound effects on maintaining the genetic integrity of a cell. This hypothesis was first proposed when a mutant version of cyclin E that had one of its Cdk2 phosphorylation sites mutated (T380A), and was shown to be semi-resistant to proteolysis, was found to induce an elevated frequency of chromosome instability compared to wild-type cyclin E (Spruck *et al*., 1999). In the present study, cytometric analysis confirmed this association in tumors, showing that all but one tumor with an *hCDC4* gene mutation and all tumors with phosphorylated cyclin E displayed a polyploid or polyploidy/aneuploid DNA profile. These results are consistent with previous *in vitro* studies that demonstrated an association of deregulated cyclin E changes in cell ploidy (Haas *et al*., 1997; Spruck *et al*., 1999).

Since cyclin E abnormalities, *hCDC4* gene mutations, and chromosome instability preferentially associate with undifferentiated high-grade tumors, we repeated our statistical analysis using exclusively grade III tumors to avoid bias. Elevated cyclin E protein (low *vs* intermediate/high levels) was found not to be significantly associated with chromosomal abnormalities, based on the five probes tested ($P = 0.29$). The presence of *hCDC4* mutations still showed borderline significance ($P = 0.073$), whereas phosphorylated cyclin E still was significantly associated ($P = 0.004$) with severe chromosomal alterations. The finding that chromosome instability was significantly associated with markers of altered cyclin E degradation but not elevated cyclin E protein is intriguing. The absence of a significant correlation between elevated cyclin E and chromosome alterations in tumors could reflect the fact that aneuploidy in the present study was assessed at only five independent loci, and only two of these loci represented LOH hotspots. Our previous study describing an increased frequency of aneuploidy and polyploidy in ectopically expressing cyclin E cells used a more sensitive karyotypic analysis (Spruck *et al*., 1999). Alternatively, these results may suggest that

Table 1 Grade, Cyclin E protein level and phosphorylation status, *hCDC4* mutation, FISH and cytometry data for 28 endometrial adenocarcinomas

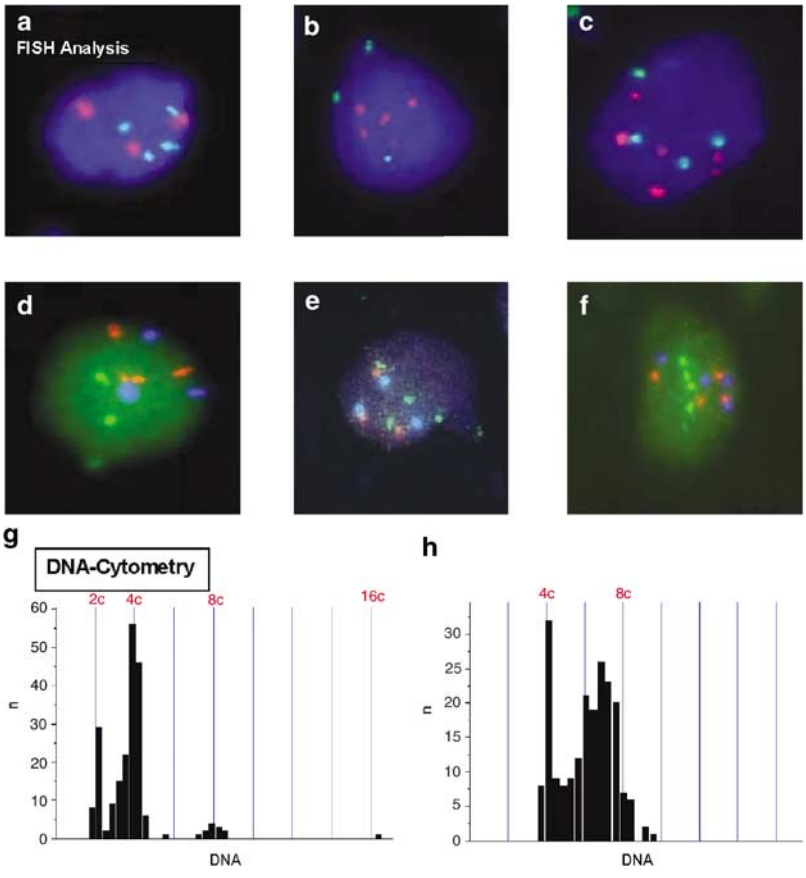
					FISH analysis					
No.	*Grade*	*Cyclin E protein*	*Phos-cyclin E*	*hCDC4 mutation*	*CEN1*	*CEN8*	*MYC*	*CEN11*	*IGH*	*Cytometry*
1	G3	High	+	*mut*[1]*(6)*	+ *53%*	+ *44%*	+ *40%*	+ *50%*	+ *48%*	Tetraploid/aneuploid
2	G3	Intermed	+	*mut(9)*	+ *17%*	+ *27%*	+ *25%*	+ *22%*	+ *35%*	Tetraploid
3	G3	Low	+	*mut(11)*	+ *11%*	– 8%	+ *48%*	+ *14%*	– 17%	Tetraploid
4	G3	High	+	*mut(6)*	+ *46%*	+ *55%*	+ *55%*	+ *44%*	+ *60%*	Tetraploid/aneuploid
5	G3	Intermed	+	*mut(2spl)*	+ *34%*	+ *27%*	+ *28%*	+ *23%*	– 10%	Aneuploid
6	G2	Low	+	*mut(8)*	+ *43%*	+ *46%*	+ *47%*	+ *27%*	– 10%	Aneuploid
7	G3	Intermed	+	wt[2]	+ *14%*	+ *79%*	+ *73%*	+ *16%*	– 10%	Tetraploid
8	G3	Low	+	wt	+ *28%*	+ *42%*	+ *15%*	+ *40%*	+ *30%*	Aneuploid
9	G3	High	–	*mut(alpha)*	+ *47%*	+ *84%*	+ *87%*	– 5%	– 9%	Tetraploid
10	G3	Intermed	–	*mut(beta)*	+ *33%*	+ *18%*	+ *18%*	+ *34%*	+ *30%*	Tetraploid/aneuploid
11	G3	Intermed	–	*mut(beta)*	– 10%	– 9%	– 9%	– 9%	– 14%	Na
12	G3	Low	–	wt	– 7%	– 8%	+ *13%*	– 3%	– 20%	Aneuploid
13	G3	High	–	wt	– 4%	– 5%	– 4%	– 8%	– 8%	Aneuploid
14	G3	Low	–	wt	– 4%	– 2%	+ *62%*	– 8%	+ *55%*	Diploid
15	G2	Low	–	wt	+ *15%*	– 1%	– 1%	– 1%	– 6%	Diploid
16	G3	Intermed	–	na[3]	– 1%	– 2%	– 2%	– 1%	– 11%	Diploid
17	G3	Low	–	wt	– 6%	– 1%	– 0%	– 5%	– 10%	Diploid
18	G3	Intermed	–	na	– 2%	+ *57%*	+ *58%*	+ *47%*	+ *42%*	Aneuploid
19	G2	Low	–	wt	– 5%	– 4%	– 2%	– 3%	–18%	Na
20	G2	Low	–	wt	– 2%	– 2%	– 2%	+ *10%*	– 7%	Na
21	G2	Low	–	na	× ***34%del***	– 4%	– 4%	× ***23%del***	– 7%	Na
22	G2	Low	–	na	+ *24%*	+ *13%*	+ *13%*	+ *20%*	– 14%	Na
23	G3	Low	–	wt	+ *60%*	– 4%	– 8%	– 5%	– 7%	Na
24	G2	High	–	wt	+ *37%*	+ *68%*	+ *73%*	– 9%	– 23%	Na
25	G2	Low	–	na	– 6%	– 3%	– 8%	– 4%	– 14%	Na
26	G2	Intermed	–	wt	+ *19%*	× ***15%del***	+ *31%*	+ *14%*	– 22%	Na
27	G2	Low	–	na	+ *23%*	– 3%	– 6%	– 4%	– 16%	Na
28	G3	Low	–	wt	+ *35%*	+ *23%*	+ *23%*	– 4%	– 11%	Na

[1]mut = mutation, exon is specified in brackets; [2]wt = wild type; [3]na = not available. In all, 28 fresh frozen and 17 paraffin-embedded endometrial adenocarcinoma specimens were analysed. Cyclin E protein levels and phosphorylation status were determined by Western blot analysis and reported previously (Spruck *et al*., 2002). Levels were divided into three groups according to signal intensities (low, intermediate, and high). *hCDC4* gene mutational analysis was determined by SSCP analysis and also reported previously (Spruck *et al*., 2002). FISH analysis shows the hybridization results of five probes corresponding to four different chromosomes. A total of 100 nuclei were analysed. +: amplifications; ×: deletions; –: no aberration (e.g. four aberrant hybridzation signals on every single locus on the five chromosomal regions are strong indicators for a tetraploid cell). FISH probes used are: cen1, centromere chromosome 1; cen 8, centromere chromosome 8; myc, chromosome 8q24; cen 11, centromere chromosome 11; IGH, chromosome 14q32. Ploidy results were confirmed by cytometric analysis. Degree of chromosomal aberration is shown in percentage (cutoff values: >26% for IGH, >10% for MYC, and >9% for CEP1, CEP8, and CEP11). Italic values FISH analysis indicate a gain of signals, whereas bold italics indicate a loss of chromosomal material. For materials and methods for FISH analysis and cytometry, see Figure 1

deregulation of cyclin E expression relative to cell cycle progression may be more important than simple overexpression in contributing to human tumorigenesis. In support of this hypothesis, we previously found that deregulated expression of a mutant form of cyclin E (T380A) that was semi-resistant to hCdc4-mediated degradation induced a higher degree of aneuploidy and polyploidy compared to wild-type cyclin E (Spruck *et al*., 1999). The absence of significant chromosome instability in tumors containing elevated cyclin E, and lower instability in the wild-type cyclin E expressing cells in our previous study, could reflect the fact that the level of cyclin E expression in these cells may not be sufficient to overcome SCF^{hCdc4} -mediated degradation, and cyclin E protein levels are likely to remain periodic at the G_1–S phase boundary.

The molecular mechanism by which deregulated cyclin E contributes to chromosome instability is not understood. Although the targets of cyclin E/Cdk2 phosphorylation are not well defined, cyclin E has been implicated in the regulation of at least two processes that are central to the faithful transmission of chromosomes – establishment of competent pre-replication complexes (Coverley *et al*., 2002) and centrosome duplication (Lacey *et al*., 1999). In *Xenopus*, deregulated expression of cyclin E has been shown to interfere with the establishment of pre-replication complexes (Coverley *et al*., 2002), and an equivalent effect in mammalian cells has been suggested (manuscript submitted). If hCdc4 is functionally inactivated by mutation, cyclin E/Cdk2 kinase activity might persist into subsequent downstream cell cycle phases. In support of this, we have performed immunohistochemical staining on endometrial adenocarcinomas and found that cyclin E protein is deregulated relative to the cell cycle in tumors that contain *hCDC4* gene mutations (manuscript in preparation). The persistence of cyclin E-Cdk2 in later cell cycle phases could function to inappropriately phosphorylate proteins involved in pre-replication complex assembly and/or centrosome duplication, or other processes, thus negatively impacting the fidelity of chromosome transmission and ultimately leading to genomic instability

The contribution that deregulated cyclin E makes to genomic instability in human tumors remains to be determined. Cyclin E abnormalities have been reported in many types of human tumors; however, the vast majority of these studies have only made an evaluation on the level of cyclin E protein and have not defined whether cyclin E is deregulated as a function of cell cycle progression. Functional inactivation of *hCDC4* may represent a major pathway leading to cyclin E deregulation in tumors. In support of this, *hCDC4* gene mutations have been reported in primary endometrial



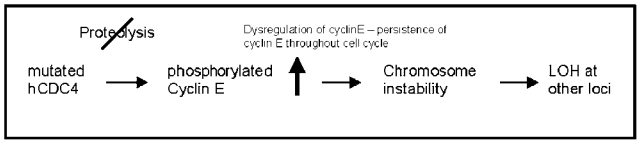


Figure 2 Proposed molecular pathway for contribution of deregulated cyclin E to chromosome instability during tumorigenesis. Functional inactivation of *hCDC4* by mutation and coordinate LOH results in an altered degradation and stabilization of cyclin E protein. The persistence of cyclin E throughout the cell cycle likely interferes with downstream cell division processes that influence the fidelity of chromosome transmission. The resultant chromosome instability could unmask recessive alleles, such as in tumor suppressor genes that function to drive the progression of tumors to more advanced stages of malignancy

tumors as well as tumor-derived cell lines of the breast and ovary (Moberg *et al*., 2001; Strohmaier *et al*., 2001; Spruck *et al*., 2002). Furthermore, *hCDC4* is localized to chromosome 4q32, a region deleted in approximately 30% of all human tumors (Knuutila *et al*., 1999). *hCDC4* could function as a tumor suppressor by maintaining the normal periodicity of cyclin E protein at the G_1–S boundary. Functional inactivation of hCdc4 likely leads to a deregulation of cyclin E relative to the cell cycle driving chromosome alterations. In turn, this instability could function to uncover recessive mutations in other growth regulatory genes, contributing to tumorigenesis (Figure 2).

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Figure 1 Representative examples of FISH (**a–f**; tissue samples #2 and #10, see Table 1) and cytometry (**g**, **h**) analysis of endometrial tumors. Hybridization of CEP1 (red signal) and CEP11 (green signal) probes shows **(a)** trisomy for chromosome 1 and tetrasomy for chromosome 11, **(b)** tetrasomy 1 and trisomy 11, and **(c)** hexasomy 1 and tetrasomy 11. Hybridization of CEP8 (blue signal), MYC (red signal), and IGH (green signal) shows **(d)** trisomy 8 and disomy 14, **(e)** trisomy 8 and tetrasomy 14, and **(f)** possible tetraploidy. All tumor samples shown have *hCDC4* mutations and/or phosphorylated cyclin E. For FISH analysis, single nuclear suspensions were prepared from aliquots of frozen tumor tissue fixed for 2–3 h in 1% paraformaldehyde solution (1 ml). Samples were washed in 0.9% NaCl, digested in 0.005% proteinase K for 30–60 min at 37°C, and mechanically disaggregated by pressing through a nylon mesh. Nuclei were sedimented by centrifugation, washed and suspended in phosphate-buffered saline (PBS), spotted onto microscope slides, and air-dried. Slides were washed for 30 min in 2 × SSC at 37°C and dehydrated in ethanol prior to hybridization. Cytogenic analysis of interphase nuclei was performed using probe sets corresponding to loci on chromosomes 8q24, 14q32 and centromere of chromosome 8 (LSI IGH/MYC, CEP8), 1q12 (CEP1), and 11p11.11-q11 (CEP 11 [D11Z1]). All probes are commercially available (Vysis, IL, USA). Hybridization was performed according to the manufacturer. Analysis was performed using an epifluorescence microscope connected to a charged coupled device (CCD) camera. In all, 100 nuclei were enumerated for each tissue sample. Each FISH probe was tested on 20 normal endometrial tissue samples to determine the frequency of false positives. Greater than two fluorescence signals per interphase nuclei were observed at a frequency of 8% for the IGH probe (s.d. = 6%) and 4% for the MYC (s.d. = 2.1%), CEP8 (s.d. = 2.0%), CEP1 (s.d. = 2.2%), and CEP11 (s.d. = 1.8%) probes. The cutoff levels for the five probes defined as the mean of false positives plus three s.d.s was 26% for IGH, 10% for MYC, CEP8, CEP1, and 9% for CEP11. All samples were hybridized and scored by two independent investigators in a blinded study. **(g)** DNA cytometry showing a tetraploid DNA histogram of a sample with *hCDC4* mutation. At 4c and 8c a peak can be observed. Corresponding FISH examples for this tumor are shown in (**b**), (**c**), and (**f**) above. (**h**) DNA histogram showing an aneuploid (7c) and tetraploid DNA content. Corresponding FISH analysis for this tumor is shown in (**a**), (**d**), and (**e**) above. Tumors contain elevated levels of phosphorylated cyclin E and an hCDC4 gene mutation. Nuclear DNA measurements were performed by image cytometry. For cytometric analysis, single cell preparations were prepared. In brief, representative tissue blocks from the formalin-fixed and paraffin-embedded surgical specimens were sectioned (two 50 μm thick sections from each tumor block) and nuclei were isolated using the protocol of Mikuz *et al*. (1985). Prior to staining, the cytospin preparations were air-dried for 1 h, fixed in 4% paraformaldehyde for 30 min, and rinsed in distilled water. Single nuclei preparations were stained according to the Feulgen procedure using the CAS 200 staining kit (CAS Quantitative DNA Staining Kit, Becton Dickinson, Cellular Imaging Systems, San Jose, CA, USA). At least 100 endometrial cancer cell nuclei were examined for each specimen. Imaging was performed using a Reichert Univar Microscope equipped with a 100 × oil immersion objective (n.a. 1.34), a Hitachi b/w CCD camera KP-M1 (pixel size 121 μm^2), a PCVision Plus 8-bit 512 × 512 pixel frame grabber board, and a custom-made image analysis system based on the OPTIMAS(R) Software package. The images were densitometrically measured. For densitometric measurements, the microscope was equipped with a narrow band bypass filter of 610 (±10) nm. The system was designed to fulfill all criteria of the *European Society of Analytical Cellular Pathology* (*ESACP*) consensus report (Haroske *et al*., 2001). Karyotype histograms were created using Origin Graphics Software (Microcal Origin, Microcal Software, Inc., MA, USA). DNA index (DI) was calculated according to Böcking *et al*. (1994). A DI of 1 corresponds to diploidy (2c). The ploidy of each tumor specimen was expressed as the DI, defined as the median integrated optical density (IOD) divided by the mean IOD of the reference cells. At least 20 fibroblasts from each preparation were selected by an experienced pathologist after imaging and served as internal reference cells. Fibroblasts were identified microscopically because of their cigar-formed shape of the nuclei. Histograms were classified as diploid, tetraploid, or multiploid based on the following criteria: diploid histograms demonstrate a distinct G_0/G_1 peak at 2C (DI = 1 ± 0.1) with a small proportion of nuclei in S and G_2/M (4C) phases; tetraploid histograms demonstrate distinct 2C (DI = 1±0.1) and 4C (DI = 2±0.2 with >20% of total cells at 4C) peaks; aneuploid histograms demonstrate scattered DI distributions or uni-, bi- or multi-modal DI distributions that were not diploid or tetraploid. Aneuploid/tetraploid tumors designate a class of aneuploid tumors that also display a pronounced 4C peak

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