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Tumorigenesis and Neoplastic Progression

Genomic Amplification of the Human Telomerase Gene (*TERC*) in Pap Smears Predicts the Development of Cervical Cancer

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Invasive cervical carcinomas almost invariably carry extra copies of chromosome arm 3q, resulting in a gain of the human telomerase gene (*TERC*). This provided the rationale for the development of a multicolor fluorescence *in situ* hybridization (FISH) probe set as a diagnostic tool for the direct detection of *TERC* gains in Pap smears. We previously used this probe set to show that cervical intraepithelial neoplasia (CIN) 2 and CIN3 lesions could be distinguished from normal samples, atypical squamous cell of undetermined significance (ASCUS) and CIN1, with a sensitivity and specificity exceeding 90%, independent of the cytomorphological assessment. In the current study, we explored whether gain of 3q and amplification of *TERC* could predict progression from CIN1/CIN2 to CIN3 and invasive carcinoma. We applied our probe set to a series of 59 previously stained Pap smears for which repeat Pap smears and clinical follow-up were available. The samples included CIN1/CIN2 lesions that progressed to CIN3 (progressors), CIN1/CIN2 lesions that regressed spontaneously (regressors), and normal Pap smears from women who subsequently developed CIN3 or cervical cancer. Here, we show that progressors displayed a gain of 3q whereas none of the regressors showed this genetic aberration. These data suggest that 3q gain is required for the transition from CIN1/CIN2 to CIN3 and that it predicts progression. Of note, 3q gain was found in 33% of cytologically normal Pap smears from women who were diagnosed with CIN3 or invasive cervical carcinoma after a short latency. The sensitivity of our test for predicting progression from CIN1/CIN2 to CIN3 was 100% and the specificity, ie, the prediction of regression, was 70%. We conclude that the detection of 3q gain and amplification of *TERC* in routinely collected Pap smears can assist in identifying low-grade lesions with a high progression risk and in decreasing false-negative cytological screenings. *(Am J Pathol 2005, 166:1229–1238)*

The visualization of chromosomal aneuploidy and copy number changes of specific cancer-associated genes by FISH has become an important complement to routine morphological assessment of cytological samples.[1] This approach is biologically valid and successful because chromosomal aneuploidy and the resulting genomic imbalances are specific for cancer cells, distinct for different carcinomas, and occur early during disease progression.[2,3] Some genomic imbalances are correlated with poor prognosis and treatment failure,[4–6] and others, such as amplification of the *Her2/neu* oncogene in breast cancer, can guide therapeutic decisions.[7] These data provided the rationale for the development of a three-color probe set as a genetic test for the diagnosis of cervical cancer in routine cytological samples.[8] Like most other human carcinomas, cervical cancers are defined by a conserved distribution of genomic imbalances. In addition to infection with high-risk human papilloma virus,[9,10] the sequential transformation of cervical squamous epithelium requires the acquisition of additional copies of chromosome arm 3q,[11] among other cytogenetic abnormalities.[12] Using a genomic probe for the *TERC* gene on chromosome band 3q26 in combination with two control probes (CEP3 and CEP7), we were able to show that copy number increases of this locus precede malignant conversion of dysplastic lesions to invasive carcinomas, and accompany the gradual transition

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Table 1. Comparison of Terminology: Cytological Classification Systems

Bethesda classification	Cervical intraepithelial neoplasia (CIN)	Pap I-V (used in Germany)
Normal	Normal	Pap I, Pap II
ASCUS	ASCUS	Pap IIw, Pap III
LSIL (low-grade squamous intraepithelial lesion)	CIN 1	Pap IIID
HSIL (high-grade squamous intraepithelial lesions)	CIN 2	Pap IIID
HSIL	CIN 3	Pap IVa, Pap IVb
Carcinoma	Carcinoma	Pap V

ASCUS, atypical squamous cell of undetermined significance.

from ASCUS and CIN1 to CIN2 and CIN3. Therefore, the application of this probe set provides an objective genetic test for the diagnosis of cervical dysplasia. Because it is extremely difficult to collect clinical material that would allow the assessment of whether a gain of *TERC* is associated with an increased risk of progression of CIN1/CIN2 to CIN3 and to invasive disease, and because it is ethically impossible to design randomized clinical trials that would establish whether additional copies of *TERC* would expedite progression from CIN2 and CIN3 to an invasive carcinoma, we have designed a retrospective study. We identified 59 previously stained and routinely diagnosed Pap smears from 34 patients who were assigned to three groups: 1) 12 CIN3 lesions, for which a previous Pap smear was evaluated as CIN1 or CIN2; 2) 10 CIN1 or CIN2 lesions, for which a subsequent Pap smear showed no dysplastic cells; and 3) 12 normal Pap smears from women who were diagnosed with CIN3 or invasive disease after a follow-up of only 1 to 3 years. Cytological images of all samples were recorded before the hybridization of the cervical cancer-specific FISH probe set. Our goal was to address the following questions: does detection of additional copies of *TERC* in CIN1 and CIN2 lesions allow one to distinguish between lesions with and without progression to *in situ* or invasive malignancy, and would the use of our genetic test on normal Pap smears have predicted the occurrence of cancer in a certain percentage of the initially negative cytological evaluations?

Materials and Methods

Patient Samples and Cytological Screening

Three groups of samples were collected from the archive of the Laboratory of Cytopathology at the Klinik Kloster Paradiese in Soest, Germany, with informed consent. The Pap smears were evaluated according to established routine diagnostic procedures, ie, initial screening by a cytotechnologist, and when aberrant cells were found, a consensus diagnosis by two cytopathologists. Cytological grading was performed according to a custom classification system in Germany. Table 1 presents the conversion of the German classification system (based on the Munich nomenclature)[13] to the Bethesda nomenclature[14] and the nomenclature using cervical intraepithelial neoplasias. The first patient group ($n = 22$) comprised patients with Pap smears that were assessed as CIN3 (PapIV), who had earlier Pap smears diagnosed as CIN1/CIN2 (PapIIID). The second group ($n = 19$) consisted of patients with Pap smears assessed as CIN1/CIN2 who had normal Pap smears on follow-up. The third group ($n = 23$) consisted of patients with Pap smears assessed as CIN3 (Pap IVa/b) or carcinoma (PapV), for whom previous Pap smears were normal. All samples had been stained according to standard procedures and were embedded in a permanent mounting medium under coverslips. We first acquired cytological images from all samples. From cytologically normal lesions, 15 to 30 bright-field images were taken from areas on the slides that contained epithelial cells with reasonable cell density. If during the screening process of normal Pap smears, cells that appeared suspicious were encountered, images were taken of these as well. From the CIN lesions and the carcinomas, between 15 and 30 images were acquired from areas that contained phenotypically suspicious cells using a ×20 Leica Phase contrast dry objective (NA 0.5) (Leica, Wetzlar, Germany). The *xy* coordinates of these areas were recorded. The coverslips were then removed using xylene and the slides were prepared for *in situ* hybridization (see below). After relocation, FISH images were acquired using a ×40 Leica oil immersion objective (NA 1.25). To acquire images for the identical area on the slides, several fluorescent images were taken. Successful analysis of sequential samples was possible in 12 of 22 cases in group 1, in 10 of 19 cases in group 2, and in 12 of 23 cases in group 3. The percentage of successfully hybridized samples was considerably lower than in the series of fresh, routinely prepared cytological samples in our previous study.[8] This was expected because the Pap smears used here were stained, fixed, and permanently embedded in mounting medium. The morphological images that were acquired before FISH were reviewed and evaluated independently by two cytopathologists to ensure a direct correlation of the genetic with the cytological diagnosis. In the few cases of minor discordance between the two pathologists a consensus diagnosis was achieved. The few cases of discordance between initial and review diagnosis are specified in the Results section and in Tables 2 to 4.

FISH and Signal Enumeration

Before *in situ* hybridization the coverslips were removed by incubating the slides in xylene for 2 to 4 days. After removal of the coverslips, the slides were washed twice in xylene, rehydrated, and destained in 0.5% HCl/70% EtOH for 1 to 2 hours. Slides were pretreated with 0.05% pepsin for 10 to 30 minutes and fixed in 100% EtOH. The three-color fluorescent probe panel has been previously described in detail.[8] It consists of a BAC contig that

Table 2. Group 1: Progressors with 3q-Positive PAP IIID

Case no.	Date of birth	Date of Pap IIID	Review diagnosis (consensus diagnosis of two pathologists evaluating the Pap images corresponding to the areas that hybridized with 3q)	Hybridization patterns observed in the Pap IIID and the number of nuclei counted for each pattern. Patterns are described in the following order: CEP7-CEP3-3q	3q status	Date of Pap IVa	Review diagnosis (consensus diagnosis of two pathologists evaluating the Pap images corresponding to the areas that hybridized with 3q)	Hybridization patterns observed in the Pap IVa and the number of nuclei counted for each pattern. Patterns are described in the following order: CEP7-CEP3-3q	3q status
1	1/31/68	Oct. 00	Pap IVa, CIN3	1× 2-2-4, 1× 2-2-3, **3× ?-7-3, 1× 3-3-3**, 1× 5-5-5, 1× ?-?-5	Gain	Dec. 00	Pap IVa, CIN3	**5× 2-3-3**, 1× 3-3-3, 1× 3-4-4, 1× 4-5-5	Gain
2	12/13/51	Aug. 00	Pap IIID, CIN 2	**14× 2-2-3**, 1× 2-3-3, 13× 3-3-3	Gain	Oct. 00	Pap IVa, CIN3	**9× 2-2-3,** 3× 2-3-3, 14× 3-3-3	Gain
3	3/28/72	Jun. 01	Pap IIID, CIN 2	3× 2-3-3, **2× 2-3-3 or 4, 2× 2-3-4**, 3× 2-4-4, 2× 3-3-3	Gain	Sept. 01	Pap IVa, CIN3	**53× 2-2-3, 4× 2-3-3, 4× 2-3-4**, 1× 2-2-5, 5× 3-3-3	Gain
4	1/22/68	Jan. 01	Pap IIID, CIN 2	**6× 2-2-5, 18× 2-2-5**, 4× 2-7-4 or 5	Gain	Mar. 01	Pap IVa, CIN3	**5× ?-?-6, 4× ?-?-5 or 6**	Gain
5	9/22/63	Jun. 00	Pap IIID, CIN 2	8× ?-2-2, **18× ?-3-4**, 5× 7-2-4, 6× ?-?-4	Gain	Jul. 01	Pap IVa, CIN3	9× 2-2-2, 1× 2-2-3, 3× 2-3-3, 2× 2-2-4, **5× 2-3-4**	Gain
6	10/26/58	Jul. 00	Pap IIID, CIN 2	20× 2-2-2, 2× 2-2-3, **2× 2-3-4, 9× 3-4-4, 4× 3-4-5**	Gain	Sept. 00	Pap IVa, CIN3	1× ?-?-2, **2× ?-?-4**, 2× 2-?-5, **11× 3-?-5, 2× ?-?-5**, 1× 3-7-8	Gain
7	7/6/64	Oct. 00	Pap IIID, CIN 2	10× 2-2-2, 12× 2-3-3, 2× 2-4-4	Gain	2003	Pap IVa, CIN3	n.d.	n.d.
8	1/6/65	Dec. 97	Pap IIID, CIN 2	11× 2-2-2, 1× 2-2-3, 1× 2-4-4, **8× 4-4-4**, 1× 4-4-5	Tetraploid	Feb. 96	Pap IVa, CIN3	1× 3-3-3, 1× 3-4-4, **7× 4-4-4**, 1× 4-4-5, **4× 4-5-5**, 2× 5-5-5	Gain
9	74	1997	Pap IIv, ASCUS	2× 2-2-2, 1× 2-2-4, **4× 4-4-4**	Tetraploid	Jan. 99	Pap IVa, CIN3	2× 2-2-2, 2× 3-4-4, **15× 4-4-4**, 1× 4-4-5, **2× 4-4-4 or 5, 2× 4-5-5**, 1× 4-4-6, 1× 5-5-5	Gain
10	6/12/68	Oct. 98	Pap IIID, CIN 2	**42× 4-4-4**, 1× 3-3-3?	Tetraploid	Dec. 99	Pap IVa, CIN3	**17× 4-4-4, 5× 4-5-5**, 1× 5-5-5	Gain
11	12/29/61	Mar. 97	Pap IIID, CIN 2	5× 2-2-2, **5× 4-4-4**	Tetraploid	Jul. 97	Pap IVa, CIN3	13× 2-2-2, **6× 4-4-4**	Tetraploid
12	9/25/65	1999	Pap IIID, CIN 2	2× 2-2-2, **23× 4-4-4**, 1× 4-4-4 or 5	Tetraploid	Jul. 01	Pap IVa, CIN3	4× 2-2-2, **17× 4-4-4**, 1× 4-4-5?	Tetraploid

Patient case number, date of birth, Pap smear dates, hybridization patterns, review diagnosis, and 3q status for the Pap IIID and the subsequent Pap IVa of the patient.
"Main patterns" are marked in bold.
"n.d." not determined.
Shown are the hybridization patterns and number of cells with a specific hybridization pattern observed in individual Pap smears. The column "3q status" reflects the interpretation of the hybridization patterns as it pertains to 3q copy numbers compared to the ploidy of the cells. A case was considered tetraploid when more than 20% of the cells showed a 4-4-4 pattern. A case was marked with gain when more than 20% of the cells showed a pattern within a 3q signal number greater than 2, excluded cells with 4-4-4 pattern. The number in front of the observed patterns indicates the frequency with which this pattern was observed (for instance we observed a 2-2-3 hybridization pattern in 14 cells in case no. 2).

contains the human telomerase gene (*TERC*, labeled with Spectrum Orange), a centromere enumeration probe for chromosome 3 (CEP3, labeled with Spectrum Green), and a control probe for the centromere of chromosome 7 (CEP7, labeled with Spectrum Aqua). The probe set is depicted schematically in Figure 1. Details of the hybridization conditions and posthybridization washes were previously described.[8] These probes were provided by Vysis, Inc./Abbott Laboratories (Downers Grove, IL). Approximately one-third of the samples were hybridized using a probe cocktail provided by Cancer Genetics, Inc. (Milford, MA). In this probe set the Spectrum Orange-labeled *TERC* probe was replaced with a BAC contig specific for *TERC* that was directly labeled with rhodamine using the protocol developed by Kreatech (*http://www.kreatech.com*). The performance of the probe sets was comparable (data not shown). After hybridization, the cell nuclei were counterstained with 4,6-diamidino-2-phenylindole and embedded in an anti-fade solution. Details of the FISH procedure can also be retrieved at *http://www.riedlab.nci.nih.gov*. Different numbers of cells were evaluated per case: this number was dependent on cell density and on the number of morphologically aberrant cells (as identified by previous Pap staining). Signal enumeration was primarily focused on those cells that appeared suspicious by routine cytological screening (see examples in Figure 2). The signal enumeration procedure therefore differed from the one previously described.[8] Cases were considered positive for the 3q assay when more than 20% of the cells exhibited a *TERC* signal number greater than 2. This threshold is higher than the one in our first report,[8] however, increasing the threshold was necessary because the enumeration procedure was different and because the hybridization quality was inferior, which is attributable to the use of previously stained, archived, and permanently embedded material.

Statistical Evaluation

The Fisher's exact test was used for 2 × 2 contingency table analysis of the categorical data. The two categorical

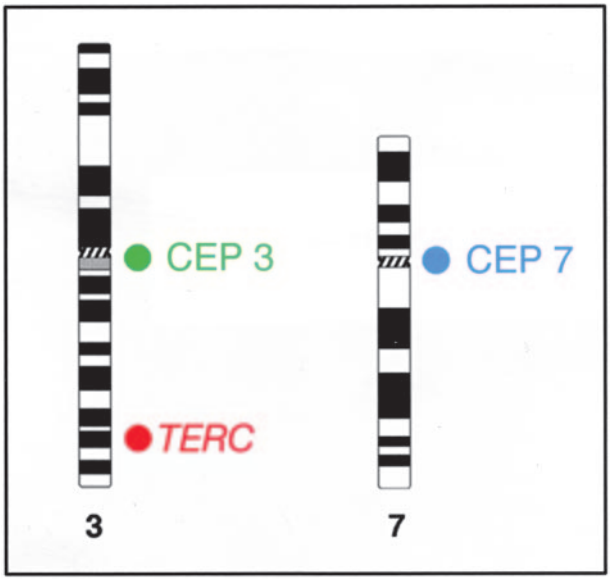


Figure 1. Triple-color FISH probe set (see Heselmeyer-Haddad and colleagues[8]).

variables used are the pathological assessment (progression and regression) and the detection of genomic aberrations, which is either positive or negative. Each cell in Table 5 reflects the observed outcomes from patient samples. The null hypothesis (H_0) postulates that the presence of genomic aberrations (either the gain of 3q or tetraploidy) and the progression status are independent from one another. The progression rate is defined as the number of cases that progress over the total number of cases tested. The upper and lower endpoints of the exact confidence intervals for estimation of this binomial parameter were denoted as $P_L(\alpha)$ by $P^{\alpha}_{L}(n,B)$ and $P_U(\alpha)$ by $P^{\alpha}_{u}(n, B)$ and were determined based on the equations below.[15]

$$P^{\alpha}_{L}(n, B)\,\frac{B}{B + (n - B + 1)\mathrm{f}_{\alpha/2,2(n-B+1),2B}}$$

and

$$P^{\alpha}_{u}(n, B) = 1 \cdot P^{\alpha}_{L}(n, n - B)$$

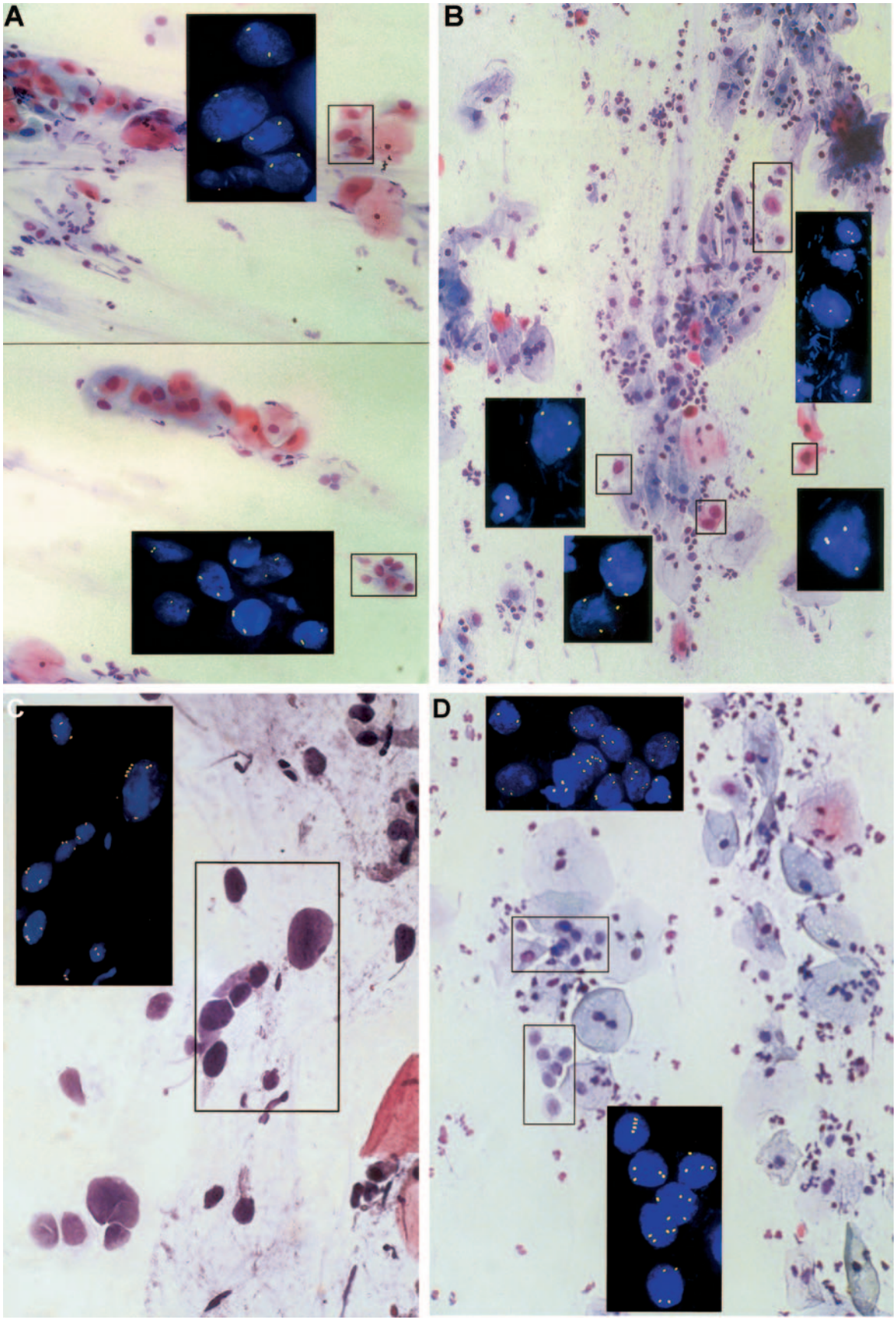
B refers to the number of progressions (successes) in the n Bernoulli trials and $f_{\gamma,n1,n2}$ is the upper γ^{th} percentile for the F distribution with n_1 degree of freedom in the numerator and n_2 degrees of freedom in the denominator.

Receiver operator characteristic (ROC) analysis was used to further establish optimal thresholds and identify FISH parameters that best predicted progression. ROC curves were generated by plotting the sensitivity for predicting progression versus 1 minus the specificity for predicting regression, calculated at percent cell thresholds ranging from 0 to 100% (1% increments). Curves were generated based on the percentage of tetraploid cells (4-4-4 hybridization pattern), the percentage of cells with 3q gain (>2 *TERC* signals per cell, excluding tetraploidy), and the percentage of cells with either tetraploidy or 3q gain (ie, >2 *TERC* signals per cell, including tetraploidy). In a ROC plot, curves that come closest to the ideal values of 100% sensitivity and 100% specificity (Figure 3A, top left corner of ROC graph) provide the best combination of sensitivity and specificity (assuming equal importance of each) and optimal thresholds are typically selected from points near the breaks in the curves (region closest to top left corner; curve slope near 45°). A better view of the dependence of sensitivity and specificity on threshold can be obtained by plotting the distance from ideal (DFI) versus threshold (Figure 3B). DFI is defined as the distance from the ideal point (0, 1) on the ROC plot (100% sensitivity, 100% specificity), and is calculated as $[(1\text{-sensitivity})^2 + (1\text{-specificity})^2]^{1/2}$. DFI is smallest for the best combined sensitivity and specificity (giving equal weight to each) and varies from a value of 0 for thresholds providing 100% sensitivity and 100% specificity, ie, the ideal point to a maximum value of $2^{1/2}$. Minima on a DFI curve indicate the best values for thresholds, and broad minima are indicative of more robust assays because placement of thresholds is less critical.

Results

Our findings constitute a comprehensive retrospective evaluation of 59 Pap smears in an attempt to validate amplification of the *TERC* gene as a prognostic marker in cervical cytology. This was achieved by visualization of *TERC* amplification in routinely collected and previously stained Pap smears. Correlation of cellular phenotype with the genomic makeup of cells was focused on patients with the following history: 1) the first group consisted of 12 cases for which the initial diagnosis was CIN1 and CIN2. Matched Pap smears screened 2 months to 2 years after the initial diagnosis revealed progression to CIN3. This group was selected to evaluate whether CIN1 and CIN2 lesions that progress to CIN3 already carry extra copies of chromosome arm 3q. 2) The second group included samples from 10 women whose Pap smears were assessed as CIN1 or CIN2 and whose subsequent Pap smears, several months to 2 years later,

Figure 2. A: Hybridization of the *TERC* gene (yellow) to previously stained routine Pap smears from patient 9 (group 2, regressors; Table 3). This Pap smear was assessed as Pap IIID (CIN1). Note that the morphologically suspicious cells do not carry extra copies of the *TERC* genes (two copies per cell only). Two distinct areas of the slide are visualized. For simplicity, only the signals for the *TERC* probe are shown. **B:** Hybridization of the *TERC* gene (yellow) to previously stained routine Pap smears from patient 2 (group 1, progressors; Table 2). This Pap smear was assessed as Pap IIID (CIN2). Multiple nuclei that appeared aberrant during the cytological screening throughout the slide reveal extra copies of *TERC* (shown as red signals). Note that both larger nuclei and cells with small nuclei reveal increased copy numbers for this gene (**bottom right**). **C:** Hybridization of the *TERC* gene (yellow) to previously stained routine Pap smears from patient 7 (group 1, Table 2). This patient was initially diagnosed as Pap IIID (October 2000) and was considered a regressor because subsequent Pap smears were normal (2001). However, in 2002 the follow-up Pap smear was assessed as CIN2, and in 2003 as CIN3. The patient was therefore assigned to group 1. Note multiple 3q-positive cells in the Pap smear (main pattern 2-3-3). **D:** Hybridization of the *TERC* gene (yellow) to previously stained routine Pap smears from patient 9 (group 3, Table 4). This Pap smear was repeatedly judged as morphologically normal, yet the patient presented with a CIN3 lesion after 28 months. This case revealed four, occasionally five, copies of 3q on a diploid background (ie, two signals for CEP7). Interestingly, the subsequent CIN3 lesion showed the same main pattern (2-3-4), supporting the hypothesis of clonal expansion.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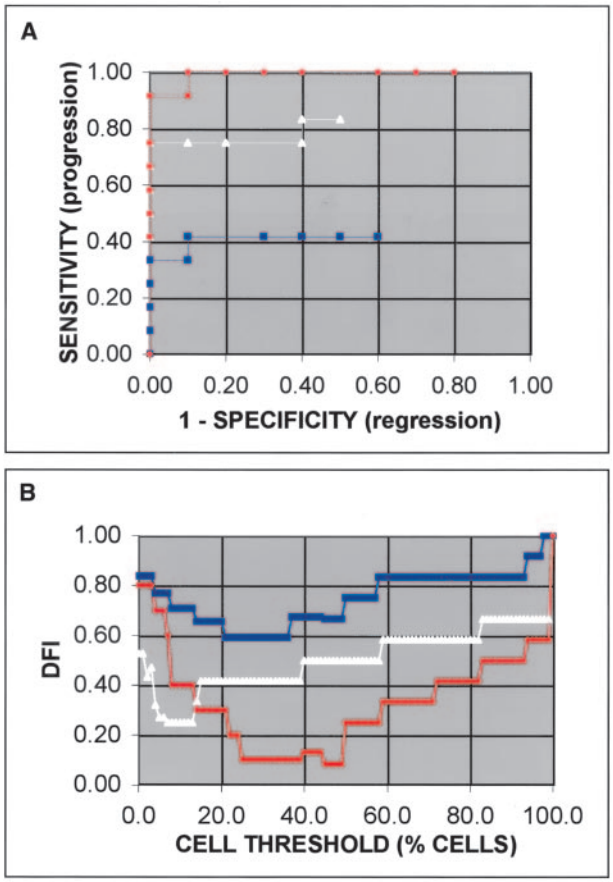

Figure 3. ROC and DFI curves for 3q gain. The **white triangles** in both **A** and **B** denote the results when considering cells with >2 *TERC* signals/cell, excluding tetraploidy as positive; the **blue squares** reflect the results when only tetraploid cell were considered, ie, hybridization patterns of four signals for each probe (4-4-4); and the **red circles** show the results when considering cells with any 3q gain (>2 *TERC* signals/cell including cells with a tetraploid hybridization pattern). **A:** ROC plot of sensitivity versus 1—specificity at thresholds ranging between 0% and 100% abnormal cells. **B:** Plot of DFI versus threshold (refer to the Materials and Methods for details).

were cytologically normal. We hypothesized that the CIN1 and CIN2 lesions in this group would not have acquired gains of 3q. 3) The third group was comprised of 12 Pap smears that were diagnosed as normal. However, in all instances, these women developed CIN3 (n = 11) or cervical carcinomas (n = 1) after a follow-up period of only 1 to 3 years. In this group we were interested to learn whether some of the normal Pap smears were actually already positive for 3q gain and to assess why cytological screening had not identified aberrant cells. The results are summarized in Tables 2 to 4 and representative images of both cytology and FISH results are displayed in Figure 2. Nine of the twelve CIN3 lesions in group 1 (Table 2, progressors) revealed varying degrees of cells with extra copies of chromosome arm 3q, and two were tetraploid (hybridization pattern of four signals for all three probes, 4-4-4); one case was not determined. Seven of the preceding matched CIN1 and CIN2 lesions were positive for 3q gain as well, indicating that those CIN1/CIN2 lesions with a high likelihood for progression frequently carry extra copies of this genetic marker. The remaining five lesions were tetraploid, including the precursors of the two tetraploid CIN3 lesions. In group 2 (Table 3, regressors), seven of the nonprogressing CIN1/CIN2 lesions were diploid (hybridization pattern of two signals each for all probes, 2-2-2); three cases were tetraploid (4-4-4), and none of the cases showed a gain of 3q. Our findings demonstrate that CIN1/CIN2 lesions that spontaneously regress do not carry a gain of *TERC*.

Eleven of the twelve CIN3 lesions and carcinomas in group 3 (Table 4, normal Pap smear followed by CIN3 or carcinoma) revealed a 3q gain. One lesion (no. 8, Table 4) was tetraploid. Of note, 4 of the 12 cytologically normal Pap smears already exhibited a gain of 3q. This suggests that the visualization of additional copies of the *TERC* gene could serve as an early and specific marker in cytologically normal Pap smears obtained from women who are prone to develop CIN3 lesions or invasive disease. In the review diagnosis of the morphological images by two experienced cytopathologists, one of these four 3q-positive lesions was upgraded from normal to CIN2 (Pap IIID), another one was upgraded to CIN3 (Pap IVa), whereas the diagnosis for two of them remained as previously determined, ie, normal. The comparison of the cytological phenotype of the cells with the genetic makeup indicates that in two cases the dysplastic cells were present on the slide, yet were indeed overlooked (a known problem in cervical cytology); however, in two other cases the cellular phenotype appeared normal on review despite the presence of chromosomal aneuploidy and gain of 3q. This demonstrates that the acquisition of specific genomic gains can precede phenotypic alterations appreciable by morphological inspection. Examples of images of these cases are displayed in Figure 2. The review diagnosis of the morphological images of the areas evaluable with the 3q marker was in concordance with the initial diagnosis for 53 of the 59 specimens (90%). Six specimens were up- or downgraded from the original diagnosis, including the two cases already discussed above (Table 4; group 3, case 10 from Pap I to Pap IIID, and case 11 from Pap II to Pap IVa). The original Pap V diagnosis of case 10 (Table 4) was downgraded to Pap IVa. In group 3, Table 4, case 6 was upgraded from Pap II to Pap IIID, and in group 1, Table 2, case 1 was upgraded from Pap IIID to Pap IVa, and case 9 was downgraded from Pap IIID to Pap IIw, ASCUS.

The statistical evaluation was based on the Fisher's exact test and the exact binomial parameter estimation, which is suited for small sample numbers. The results are summarized in Table 5. For the patients whose Pap smears contained tetraploid cells, the odds (five to three) are in favor of progression, yet the P value is 0.6749. We can therefore not reject H_0 (ie, there is no strong statistical evidence that tetraploidy is associated with progression). For cases with a 3q gain versus diploid and tetraploid cases, the P value is 0.0053. Thus, we have strong evidence to reject H_0, indicating that additional copies of 3q and progression are associated. The 95% confidence interval ranges from 0.5904 to 1.0000. Thus, with 95% confidence, we expect that the probability of progression is going to be 59 to 100%. For patients that have either a gain of 3q or tetraploidy, the P value for the

Table 3. Group 2: Regressors

Case no.	Date of birth	Date of Pap IIID	Review diagnosis (consensus diagnosis of two pathologists evaluating the Pap images corresponding to the areas that hybridized with 3q)	Hybridization patterns observed in the Pap IIID and the number of nuclei counted for each pattern. Patterns are described in the following order: CEP7-CEP3-3q	3q status	Date of Pap I/II	Review diagnosis (consensus diagnosis of two pathologists evaluating the Pap images corresponding to the areas that hybridized with 3q)	Hybridization patterns observed in the Pap II and the number of nuclei counted for each pattern. Patterns are described in the following order: CEP7-CEP3-3q	3q status
1	10/28/73	Oct. 99	Pap IIID, CIN 2	**29× 2-2-2**	Diploid	Aug. 00	Pap I, normal	34× 2-2-2	Diploid
2	9/4/63	Jan. 01	Pap IIID, CIN 2	**25× 2-2-2**, 1× 2-3-3?	Diploid	2001	n.d.	not evaluable	n.d.
3	3/29/73	2000	Pap IIID, CIN1/2	**48× 2-2-2**	Diploid	Dec. 00	n.d.	n.d.	n.d.
4	4/7/73	2000	Pap IIID, CIN 2	**48× 2-2-2**, 3× 4-4-4, 1× 4-4-4?	Diploid	2001	n.d.	not evaluable	n.d.
5	6/8/81	Jul. 99	Pap IIID, CIN 2	**49× 2-2-2**, 1× 2-2-3?, 1× 2-2-4, 2× 4-4-4	Diploid	Dec. 99	n.d.	not evaluable	n.d.
6	3/1/74	Feb. 99	Pap IIID, CIN1/2	**57× 2-2-2**, 1× 2-2-3?, 3× 2-2-4	Diploid	2000	n.d.	n.d.	n.d.
7	4/16/51	Aug. 99	Pap IIID, CIN 2	**19× 2-2-2**, 2× 4-7-4, 1× ?-?-4 or 5	Diploid	Jul. 01	Pap II, normal	20× 2-2-2	Diploid
8	10/29/72	1999	Pap IIID, CIN 1/2	18× 2-2-2, 1× 2-2-4, **4× 4-4-4, 1× 4-?-4**	Tetraploid	Apr. 00	n.d.	not evaluable	n.d.
9	8/2/58	2000	Pap IIID, CIN 1	50× 2-2-2, 1× 2-2-4?, **13× 4-4-4**	Tetraploid	2001	n.d.	n.d.	n.d.
10	9/15/77	Feb. 01	Pap IIID, CIN 2	10× 2-2-2, **7× 4-4-4, 1× 4-?-4**	Tetraploid	2001	n.d.	not evaluable	n.d.

Patient case number, date of birth, Pap smear dates, hybridization patterns, review diagnosis, and 3q status for the Pap IIID and the subsequent normal Pap smear of patient.

"Main patterns" are marked in bold.

"n.d." not determined.

Shown are the hybridization patterns and number of cells with a specific hybridization pattern observed in individual Pap smears. The column "3q status" reflects the interpretation of the hybridization patterns as it pertains to 3q copy numbers compared to the ploidy of the cells. A case was considered tetraploid when more than 20% of the cells showed a 4-4-4 pattern. Diploid cases showed a 2-2-2 pattern in more than 80% of the cells. The number in front of the observed patterns indicates the frequency with which this pattern was observed.

test is 0.0007. Therefore, we have great confidence in rejecting H_0. A significant correlation exists between additional copies of 3q and carcinoma development. We expect a 52 to 96% progression rate in patients with either 3q-positive or tetraploid samples.

Classification algorithms were further developed using ROC curves that plot sensitivity versus 1-sensitivity throughout a range of threshold values. In Figure 3A comparison of the ROC curves shows that tetraploidy alone (blue squares) is not a good indicator of progression, whereas 3q gain (defined for this purpose as >2 *TERC* signals/cell, exclusive of tetraploidy, white triangles) is a better indicator, and 3q gain including tetraploidy (any gain of 3q, red circles) shows very good ROC characteristics. The point on the latter ROC curve lying closest to the top left corner of the graph represents sensitivity and specificity values of 91.7% (11 of 12) and 100% (10 of 10), respectively, which are obtained for cell percentage thresholds of 45 to 49%. However, for identifying women likely to progress, higher sensitivity is preferred and 100% sensitivity (12 of 12) with 90% specificity (9 of 10) are achieved with thresholds ranging between 25 to 39%. To further ensure identification of likely progressors, a more conservative threshold of 20% was used in the present study. Figure 3B plots the same data used to construct Figure 3A but in terms of DFI versus threshold. These curves identify the threshold ranges providing the lowest combined sensitivities and specificities, and emphasize that 3q gain inclusive of tetraploidy (red circles) is better suited to predict progression (lowest and broadest minimum of the three curves) than tetraploidy alone (blue squares) or 3q gain alone (white triangles).

Discussion

The implementation of cervical cancer screening programs has greatly reduced disease incidence and mortality in industrialized countries.[16,17] However, a single cytological evaluation remains relatively insensitive, hence the need for frequent follow-up investigations. This is attributable to sampling or interpretation errors, and to the fact that some early lesions may not have acquired recognizable phenotypic alterations.[17] Invasive cervical carcinomas develop through increasing stages of cervical dysplasia and advance to CIN3, which is considered a bonafide precancerous lesion that requires surgical intervention. However, only ~15% of all low-grade dysplastic lesions follow this path of linear progression. The identification of markers of disease progression would therefore be of great clinical interest. HPV infection is a causative agent in the development of cervical cancers.[9,10] For that reason, the detection of HPV genomes in early lesions was pursued to aid with the discernment of lesions with low and high risk for progression. The test is sensitive indeed, because HPV-negative lesions have a very low risk of progression; however, only a minority of HPV-positive lesions actually progress. This leaves HPV

Table 4. Group 3: Progressors with 3q-Negative Pap I/II

Case no.	Date of birth	Date of Pap I/II	Review diagnosis (consensus diagnosis of two pathologists evaluating the Pap images corresponding to the areas that hybridized with 3q)	Hybridization patterns observed in the Pap I/II and the number of nuclei counted for each pattern. Patterns are described in the following order: CEP7-CEP3-3q	3q status	Date of Pap IVa/b or V	Review diagnosis (consensus diagnosis of two pathologists evaluating the Pap images corresponding to the areas that hybridized with 3q)	Hybridization patterns observed in the Pap IVa/b and the number of nuclei counted for each pattern. Patterns are described in the following order: CEP7-CEP3-3q	3q status
1	9/23/45	Pap I: 1999	Pap VII, normal	17× 2-2-2, 1× 2-2-3?, 1× 2-3-3?	Diploid	Pap IVb: Feb. 01	Pap IVb, CIN3	**4× 2-2-3**, 2× 2-3-3, 1× 3-3-3, 1× 2-3-4, 1× 2-5-5, **3× 3-5-5, 4× 4-5-5**	Gain
2	11/27/52	Pap II: 1999	Pap II, normal	14× 2-2-2, 1× 2-2-3?, 1× 2-3-3?	Diploid	Pap IVa: May 00	Pap IVa, CIN3	**5× 2-2-3, 5× 2-3-3**, 3× 2-4-4, 3× 4-4-4, 1× 5-4-4	Gain
3	7/9/62	Pap I: Oct. 96	Pap II, normal	15× 2-2-2, 2× 2-2-3?	Diploid	Pap IVa: Jan. 99	Pap IVa, CIN3	1× 2-2-2, 6× **2-2-3, 3× 2-3-3**, 1× 2-2-4, 1× 2-3-4	Gain
4	4/17/55	Pap I: Sept. 97	Pap II, normal	16× 2-2-2, 1× 2-4-4?, 1× 3-3-3?	Diploid	Pap IVa: June 98	Pap IVa, CIN3	4× 2-2-2, 2× 2-2-3, 6× **2-3-3**, 1× 2-3-4, 2× 3-3-3, 2× 3-3-4, 1× 3-3-5, 1× 4-4-4, 4× 3-3-?	Gain
5	6/15/77	Pap II: Feb. 98	Pap II, normal	34× 2-2-2, 1× 2-3-3?	Diploid	Pap IVa: Feb. 99	Pap IVa, CIN3	18× 2-2-2, 6× **2-2-3, 4× 2-3-3**, 1× 3-3-3	Gain
6	10/18/65	Pap II: March 00	Pap IIID, CIN1	22× 2-2-2, 1× 5-5-5?	Diploid	Pap IVa: Aug. 01	Pap IVa, CIN3	1× 2-2-2, 2× 3-4-4, 7× **4-4-4**, 4× **4-5-6, 9× 5-5-6**	Gain
7	12/12/48	Pap I: Jul. 01	Pap I, normal	22× 2-2-2, 2× ?-?-3?	Diploid	Pap IVb: April 02	Pap IV, CIN3	9× **7-?-3**, 4× ?-3-3, 2× **2-3-3**	Gain
8	02/28/67	Pap II: 1998	Pap II, normal	18× 2-2-2	Diploid	Pap IVa: June 00	Pap IVa, CIN3	61× **4-4-4**, 1× 2-2-3?, 3× 4-4-5?, 1× 3-4-4?	Tetraploid
9	9/24/65	Pap II: July 97	Pap II, normal	20× 2-2-2, 1× 2-3-3?, 18× 2-3-4, 2× 2-3-5	Gain	Pap IVa: March 00	Pap IVa, CIN 3	2× 2-2-2, 3× 2-3-3, **88× 2-3-4**, 1× 2-3-5, 2× 2-4-5, 4× 4-6-6	Gain
10	7/14/54	Pap I: 1996	Pap IIID, CIN 2	3× 2-2-2, 4× 2-?-3, 1× 2-2-3, **3× 2-?-4, 1× 2-2-4, 3× 2-2-5, 1× 2-2-5, 2× 2-5-5**, 1× 2-2-6, 1× 2-?-7, **1× 3-?-4**, 2× 4-4-4	Gain	Pap V: April 99	Pap IVa, CIN 3	4× 2-2-2, 1× 2-2-3, 3× 2-3-3, **8× 2-2-4, 8× 2-2-5, 8× 2-2-6**, 1× 2-2-7, 1× 2-2-8, **28× 2-4-4**, 1× 2-4-5, 1× 2-4-8, 1× 2-4-5, **13× 2-5-5**, 1× 2-4-6, 1× 2-6-9, 1× 3-3-3, **18× 3-4-4**, 1× 3-3-5, 1× 3-3-6, 1× 3-3-7, 1× 3-4-5, 1× 3-5-7, 1× 3-6-6, 3× 4-4-4	Gain
11	10/14/45	Pap II: 1997	Pap IVa, CIN 3	8× 2-2-2, **48× 2-5-5**	Gain	Pap IVa: Aug. 99	Pap IVa, CIN 3	6× 2-2-2, 5× 2-4-4, **121× 2-5-5**	Gain
12	5/19/19	Pap II: Aug. 98	Pap II, normal	52× 2-?-2, **15× 2-?-3, 3× 2-2-3**, 2× 4-?-4, **1× 4-?-8**	Gain	Pap V: Feb. 99	Pap V, Carcinoma	6× 2-2-2, **18× 2-2-3**, 1× 3-2-3, 3× 2-3-5, 1× 2-2-8, 1× 2-3-8, 1× 3-3-8, **87× 4-3-8**, 1× 4-4-5, 1× 4-4-8, 1× 5-4-8	Gain

Patient case number, date of birth, Pap smear dates, hybridization patterns, review diagnosis, and 3q status for the normal Pap smear and the subsequent Pap IVa/b. "Main patterns" are marked in bold.

Shown are the hybridization patterns and number of cells with a specific hybridization pattern observed in individual Pap smears. The column "3q status" reflects the interpretation of the hybridization patterns as it pertains to 3q copy numbers compared to the ploidy of the cells. A case was considered tetraploid when more than 20% of the cells showed a 4-4-4 pattern. A case was marked with "gain" when more than 20% of the cells showed a pattern with a 3q signal number greater than 2, excluding cells with 4-4-4 pattern. Diploid cases showed a 2-2-2 pattern in more than 80% of the cells. The number in front of the observed patterns indicates the frequency with which this pattern was observed.

testing with a low specificity,[18,19] which limits its clinical usefulness.

The genetic analysis of cervical cancer progression has revealed that, in addition to infection with HPV, the acquisition of specific chromosomal aneuploidies appears to be a mandatory event.[11] For instance, CGH analyses of cervical carcinomas have shown that more than 85% of invasive cervical carcinomas carry specific genomic imbalances that result in copy number increases of chromosome arm 3q.[5,11,20–26] The region of minimal overlap points to chromosome band 3q26, which contains the gene for the RNA component of human

Table 5. Statistical Evaluation

Aberration profile	Pathological assessment		*P* value from Fisher's test	Progression rate	95% confidence interval of progression rate	
	Progression	Regression			Lower	Upper
4-4-4	5	3	0.6749	0.625	0.2449	0.9148
2-2-2 and 3q gain	7	7		0.5000	0.2304	0.7696
3q gain	7	0	0.0053	1.000	0.5904	1.0000
2-2-2 and 4-4-4	5	10		0.3333	0.1182	0.6162
3q gain and 4-4-4	12	3	0.0007	0.8000	0.5191	0.9567
2-2-2	0	7		0.0000	0.0000	0.4096

Statistical analysis for contingency tables and confidence intervals of the progression rate. From the left, the column classifier for all three 2 × 2 contingency tables is the pathological assessment (progression and regression). The row classifiers are the detection of tetraploidy (top 2 × 2 table), gain of the chromosome 3q (center 2 × 2 table) and gain of the 3q including tetraploidy (bottom 2 × 2 table), respectively. The two-tailed *P* value for each table is derived from the Fisher's exact test. On the right, the progression rate for the different hybridization patterns was calculated based on the cell counts in the contingency tables. Its exact 95% confidence interval was obtained using the method described in the Materials and Methods section.

telomerase (*TERC*).[27] In a previous study we have developed and validated a FISH probe set that includes a BAC contig for this gene. Our data showed that the visualization of additional copies of *TERC* serves as a specific and sensitive test for the diagnosis of cervical dysplasia in routinely collected cytological samples.[8] In many instances, the cells that were positive for additional copies of 3q were located next to each other on the diagnostic slides. This suggests a clonal evolution event, in which extra copies of 3q render a growth advantage to cervical epithelial cells, which eventually results in a cell population in cervical carcinomas in which the majority of the cells are positive for 3q. This finding is compatible with the hypothesis that the 3q-imposed growth advantage reflects a point of no return during the sequential malignant transformation of cervical epithelial cells. From these data we concluded that the visualization of additional copies of the *TERC* gene in premalignant dysplastic lesions could not only be informative for the mere diagnosis of dysplasia, but could possibly provide information regarding the progressive potential of individual lesions. We also hypothesized that in some instances of cervical cancer, preceding Pap smears that were assessed as normal could reveal the presence of this genetic marker, the detection of which would have resulted in an earlier diagnosis. In the US, CIN1 lesions of the uterine cervix are not treated surgically, but their progression is closely monitored in defined intervals, and not all of these lesions progress to CIN2 and CIN3, which are treated, or to cancer.[28,29] In Germany, partly related to the different classification system used, CIN2 lesions (which are graded as PAPIIID), are not immediately treated. This afforded us the possibility to query whether those CIN1 and CIN2 lesions that progressed were positive for 3q and whether this feature would help to distinguish them from spontaneously regressing lesions. Our results show that, indeed, 7 of 12 CIN1/CIN2 lesions that progressed to CIN3 were positive for 3q (all matched CIN3 lesions carried amplified *TERC*). In strong contrast, none of the spontaneously regressing lesions showed a 3q gain. Although the case numbers are limited, our statistical analysis suggests a strong correlation between the presence of extra copies of 3q and cytological progression, and between the absence of 3q gain and regression. A certain number of cases in the groups of progressors and regressors showed signal patterns that are compatible with a tetraploidization of the genome (ie, four copies of CEP7, CEP3, and 3q, referred to as 4-4-4 in Tables 2 to 4). If these cases are included using a conservative threshold of 20%, the test achieves a sensitivity of 100% (ie, the association of progression with either tetraploidy or *TERC* gain) with a specificity of 70%, which is defined as the association of regression with the absence of 3q-positive patterns, ie, 3q gain including tetraploidy (7 of 10 cases, see Table 5). The hybridization patterns observed in the 3q-positive CIN3 lesions and their matched CIN1/2 precursors suggest that a certain chromosomal aneuploidy, once established, is maintained during tumor progression, which again suggests clonal expansion (Tables 2 to 4). It is striking that none of the 3q-positive lesions in which the gain of *TERC* occurred on a diploid background (as assessed with the copy numbers for CEP3 and CEP7) showed a tetraploid hybridization pattern at lower-grade lesions. All CIN3 lesions, for which the corresponding premalignant lesions were tetraploid, maintained tetraploidy or developed 3q gain on a tetraploid background. This observation confirms our previous results[8] from which we concluded that the gain of 3q can occur on the basis of either a diploid or tetraploid genome. This would suggest that quantitative DNA content measurement alone might not be sufficient as a diagnostic method in cervical cytology.[30] Although none of the samples in group 2 (regressors) were positive for 3q gain, three lesions were tetraploid. This implies that tetraploidization per se does not modify the genome such that progression is unavoidable. This is consistent with previous observations indicating that genome duplication can occur as a physiological response to certain environmental challenges.[30,31] Our data are also consistent with a study by Sudbo and colleagues[32] in which the progression of premalignant dysplastic lesions in the oral cavity (leukoplakia) could be predicted when the lesions were aneuploid (as determined by quantitative measurements of the nuclear DNA content), yet lesions that were tetraploid were equally likely to regress or progress (diploid lesions had an extremely low likelihood for progression).

In conclusion, based on a retrospective analysis of routinely collected cytological samples, we provide evidence that the acquisition of specific chromosomal aneuploidies that result in a gain of *TERC* is associated with progression of premalignant dysplastic lesions of the uterine cervix. Our data also suggest that the detection of such genomic imbalances in normal Pap smears would increase the sensitivity of individual cytological screenings and could therefore reduce false-negative diagnoses.

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