Correlation between p53 status, DNA ploidy, proliferation rate and nuclear morphology in breast cancer. An image cytometric study

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Abstract. The study was designed to detect differences in the nuclear morphology of tumours and tumour cell populations with different p53 expression in correlation with DNA ploidy and proliferation rate. The paraffin sections from routinely processed samples of 88 breast cancers were immunostained with the monoclonal p53-antibody DO-1. After localization and evaluation with a scoring system the sections were destained and stained by the Feulgen method. The nuclei were relocated automatically and measured by means of the image cytometry workstation. Significant differences between the tumours and tumour cell populations with different p53 expression were found in the euploid tumours as well as in the aneuploid tumours and in the breast cancers with a high proliferation rate. The breast cancers with a low immunoreactive score (IRS 1–4) differ from the negative cancers as well as from the cancers with a higher immunoreactive score (IRS 5–12). Evaluating the nuclear populations of the p53 positive cancers, there were differences in the features of the chromatin amount and distribution in the groups of the euploid breast cancers and in cancer with a high proliferation rate. In contrast, the nuclear populations of the aneuploid cancers did not show any differences in their nuclear morphology.

The results showed the different impacts of the p53 expression, DNA ploidy and the proliferation rate on the nuclear morphology in breast cancer.

Keywords: Nuclear morphology, p53 immunohistochemistry, S-phase, G2M-fraction, DNA ploidy

1. Introduction

The wild type of the p53 gene acts as a tumour suppressor gene, which controls the cell cycle at the G1-S-transition. Furthermore, the wild type of p53 can induce apoptosis [3,20,22,23]. Mutations of the p53 gene are a common event in the human carcinogenesis. The mutation of this gene should be associated with a loss of function and also with the loss of the growth suppressor function. Cells with DNA damage remain in the cell cycle, which leads to a higher genetic instability of the tumour.

The genetic instability is often reflected by a DNA aneuploidy. This fact has been confirmed in many studies describing a correlation between the immunohistochemically detected p53 and aneuploidy [4, 7,11]. Some authors also report a higher proliferation rate in p53 positive tumours [1,14,28].

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The DNA ploidy is only a net estimator of the whole genetic material of the cell populations. Changes in the function of cells like the p53 mutation and the DNA aneuploidy should be associated with changes in the nuclear morphology. These changes may be made objective by high resolution image analysis.

In image cytometric studies, differences in nuclear morphology in correlation with prognostic features were described in breast cancers [2,16,21,23,28,30]. In the past, we reported the correlation of p53 expression and the DNA ploidy as well as differences in the nuclear features in immunohistochemically characterized p53 positive and negative breast cancer cells [12,15].

The present study was designed to detect differences in the nuclear morphology in breast cancers as well as nuclear populations being different in their p53 expression in correlation with the DNA ploidy and proliferation. In particular we wanted to know whether differences in the nuclear morphology exist in tumours with a different p53 expression in correlation with the ploidy state and the proliferation rate, respectively. A further question was whether the nuclear populations with a different p53 expression differ in their structure in tumour groups, characterized by the ploidy state and the proliferation rate of the tumours.

2. Material and methods

Forty-nine p53-positive and 39 p53-negative breast cancers were investigated. The tumours were classified according to the WHO nomenclature [19]. The grade of malignancy was determined according to Scarff, Bloom and Richardson [6]. The clinicopathological data of the tumours are detailed in Table 1. The p53 detection was done employing dewaxed paraffin sections of routinely fixed samples. The sections were stained with the monoclonal antibody DO-1 (original supernatant [31], 1:50 dilution, incubation for 24 h at 4°C) in an avidin-biotin-peroxidase technique. The chromogen was 3-amino-9-ethylcarbazol (AEC). Finally, the nuclei were counterstained with haemalaun and embedded in glycerol gelatin.

The results of the immunostaining were assessed as an immunoreactive score (IRS) for the whole tumour. The score is the product of the percentage of immunostained cells (divided in 5 groups: 0, 1–10%, 10–50%, 50–80%, 80–100% positive cells) and the staining intensity (weak, moderate and strong reaction). The score ranges from 0 to 12. Breast cancers with a p53 IRS equal one or higher were considered as p53 positive tumours [26].

The image analysis was performed by means of a high resolution image cytometry workstation (Table 2). In each case, 500 nuclei were localized by means of a cytometry workstation. The staining intensity of the nuclei was categorized subjectively in 4 groups: negative nuclei (−), weakly positive nuclei (+), moderately positive nuclei (+++) and strongly positive nuclei (+++). After destaining during a 45 min 5N HCl hydrolysis the sections were stained according to the Feulgen method. The actual section thickness was measured by means of the confocal laser scanning microscope LSM-10 (Zeiss, Germany) at three different sites in order to correct some features of the the optical density for sectioning effects. The previously coded nuclei were relocated and measured by means of the cytometry workstation, which consists of an Axioplan microscope (Zeiss, Germany) equipped with a 486/66 MHz IBM compatible PC with a MFG frame grabber (Imaging Technology, USA) using a CCD TV camera XC-77 CE (Sony, Japan) and a computer controlled motor driven xy-scanning stage. The software based on the OPTIMAS image analysis system (OPTIMAS Corp., Seattle, WA, USA). Correction procedures for the section thickness, diffraction and glare were implemented in the software [17]. From each nucleus, 97 nuclear features derived from the segmented extinction image
Table 1
Clinical data

| Tumour size  | p53 positive cases | p53 negative cases |
|--------------|--------------------|--------------------|
| pT1          | 20                 | 18                 |
| pT2          | 27                 | 18                 |
| pT3          | 1                  | 1                  |
| pT4          | 1                  | 1                  |
| pTx          | 0                  | 1                  |
| Lymph node status |         |                    |
| pN0          | 23                 | 19                 |
| pN1          | 20                 | 17                 |
| pN2          | 1                  | 0                  |
| pNx          | 5                  | 3                  |
| Bloom–Richardson-grading |     |                    |
| G1           | 3                  | 8                  |
| G2           | 18                 | 19                 |
| G3           | 28                 | 12                 |
| Age          | 58.5 (range 27–87) | 59 (range 36–83)   |
| DNA-ploidy   |                    |                    |
| Peridiploid  | 3                  | 9                  |
| Peritetraploid| 21                 | 11                 |
| Aneuploid    | 25                 | 19                 |
| S-phase      |                    |                    |
| lower or equal 5% | 21             | 21                 |
| higher than 5%  | 27                 | 17                 |
| not estimated | 1                  | 1                  |
| G2M-fraction |                    |                    |
| lower or equal 5% | 20             | 18                 |
| higher than 5%  | 28                 | 20                 |
| not estimated | 1                  | 1                  |

Table 2
Technical equipment for the image cytometry system

| Instrument         | Specification                                      | Source       |
|--------------------|----------------------------------------------------|--------------|
| Microscope         | Axioplan®                                          | Zeiss        |
| Light source       | Halogen lamp 12 V, 100W                            | Zeiss        |
| Power supply       | not specified                                     | Zeiss        |
| Filter             | Interference filter 570 nm                         | Zeiss        |
| Condensor          | Condensor 0.9                                      | Zeiss        |
| Objective          | Plan NEOFLUAR × 63/1.25 oil                         | Zeiss        |
| Adapter            | C-mount without optical lenses                      | Zeiss        |
| TV camera          | XC77CE pixel size: 11 × 11 μm; 0.03 μm² in the object plane | Sony        |
| Frame grabber      | MFG                                                | Imaging Technology |
| Image analysis basic software | Optimas®                            | OPTIMAS      |

were computed on a MicroVAX 4000 computer (DEC, Maynard, MA, USA) which is connected via Ethernet to the image analysis workstation. The nuclear features describe the size and shape of the nucleus, the amount, the statistical and topological distribution of the chromatin and chromatin statistics in the “flat image” (Table 3).
Table 3
Acronyms of the cytometric features

| Acronym     | Short description |
|-------------|-------------------|
| **Features describing the nuclear shape and size** |
| FormFak    | Shape factor of the nucleus |
| VarKRad    | Variation coefficient of the contour radii of the nucleus |
| Res02      | Contour difference to a fitted ellipse |
| RVKont     | Radius ratio of the nuclear contour |
| RVKonv     | Radius ratio of the nuclear convex contour |
| KonvVerh   | Ratio of the area and the convex contour area |
| KontFl     | Area of the nucleus |
| KonvFl     | Convex contour area |
| FFKonv     | Shape factor of the convex contour |
| Loch       | Relative proportion of nuclear holes |
| ZEIKOFi    | Area according line coincidence |

| **Features describing the chromatin amount** |
| IODnc      | Integrated optical density without correction for section thickness |
| IOD        | Integrated optical density with stereological correction of section thickness |

| **Features describing the chromatin distribution** |
| nGran      | Number of coarse chromatin particles |
| MaxTextK   | Number of relative maxima of the texture curve |
| RIODHet    | Percentage of heterochromatin optical density and nuclear IOD |
| SKEWEExt   | Skewness of the optical density histogram of the nucleus |
| FS1M       | Mean extinction of the “flat image” (first moment of fine structure) |
| FS2M       | Standard deviation of the extinction of the flat image (second moment of fine structure) |
| FS3M       | Skewness of the extinction of the flat image (third moment of fine structure) |
| GT2sig     | Number of objects darker than 2×SD of the flat image extinction |
| LT2sig     | Number of objects brighter than 2×SD of the flat image extinction |
| MOD        | Optical density in the median filtered image |
| RadExt     | Relative mean radial extinction distribution |
| RadSTD     | Relative standard deviation of the radial extinction |
| AzisSTD    | Relative standard deviation of the azimuthal extinction |
| Azidiff    | Relative maximal azimuthal extinction difference |
| NucZahl    | “Number of nucleoli” |
| InvMo      | 52 invariant moments of the heterochromatin topology according to Hu |
| Node       | Number of nodes in the texture tree |
| Width      | Width of the texture tree |

| **Features describing the chromatin distribution in a median filtered image** |
| FnGran-FLT2sig | Features nGran-LT2sig in a median filtered image |
| FSTDObj       | Standard deviation of the optical density histogram of the nucleus |
| FExtObj       | Extinction range of all object thresholds |
| FExtGran      | Mean extinction of the coarse chromatin particles |
| FExtBas       | Extinction range of the lowest possible segmentation threshold |
| FExtHet       | Mean extinction of the heterochromatin |

The DNA image cytometry was performed on fine needle aspirates or imprints from fresh tumour material which were stained according to the Feulgen method. The DNA ploidy was assessed by the cytometry workstation. In each case at least 250 tumour cell nuclei and 20-30 lymphocytes as internal reference cells were measured. The DNA ploidy status of the tumours was classified as peridiploid, peritetraploid and aneuploid.

For the flow cytometry, samples of fine needle aspirates from fresh tumour material were used for the estimation of S-Phase and G2/M-fraction by the FACScan (Becton/Dickinson) with the test kit Cycle test.
For the univariate and multivariate statistical analyses which are based on the mean values and their standard deviations of the features from the nuclear populations in each staining category for each case, a self written software program REDUGD was used. The Bonferroni principle was applied for defining significance levels at \( p < 0.05 \) in the multivariate analyses. According to this principle the significance level desired for the actual discrimination \( (p < 0.05) \) has to be divided by the appropriate degree of freedom (equal the number of the uncorrelated variables).

3. Results

3.1. Comparison of the whole nuclear populations from tumours with different p53 IRS in groups stratified by their DNA ploidy and their proliferation rate

In this section, only the immunoreactive score of the whole nuclear population will be taken into consideration.

Differences between differently scored breast cancers were found in the euploid and aneuploid tumour groups. Whereas the peridiploid and peritetraploid tumours showed a higher irregularity of the chromatin distribution in the p53 positive tumours, the aneuploid cancers showed a higher degree of polymorphism in the tumours with a positive p53 reaction than their negative counterparts (Table 4).

When the whole nuclear population of the p53 positive tumours was evaluated, there was a difference between the peridiploid and the peritetraploid cancers in a feature of the chromatin distribution (SD FExtBas; higher in the tetraploid cancers). However, the p53 positive nuclei of the peritetraploid cancers showed the higher values in a feature of the nuclear size, in the number of the coarse chromatin particles and in the standard deviation of two invariant moments of the chromatin structure than the diploid cancers (Table 5).

In the p53 negative tumours, the group of the diploid and tetraploid cancers differs from the aneuploid tumours in a feature, describing a higher variability of the elliptical shape in the aneuploid tumours.

In breast cancers with a high proliferation rate (S-Phase and G2M fraction greater than 5\%, respectively), a number of significant different features were found. They describe a higher degree of polymorphism of the nuclei in the p53 positive tumours compared with the cancers without immunohistochemically detectable p53. Furthermore, the p53 positive tumours also had nuclei with irregular chromatin distribution and a variable amount of chromatin (Tables 6, 7).

Table 4

| Feature                                      | p53 negative | IRS 1–4 | IRS 5–12 |
|----------------------------------------------|--------------|---------|----------|
| Euploid tumours                              |              |         |          |
| SD percentage of heterochromatin optical density and nuclear IOD* | 0.173        | 0.231   | 0.202    |
| SD 3rd moment of fine structure              | 0.946        | 1.271   | 1.123    |
| invariant moment 22                          | 0.501 \times 10^{-4} | 0.439 \times 10^{-4} | 0.156 \times 10^{-3} |
| Aneuploid tumours                            |              |         |          |
| SD integrated optical density (without correction of section thickness) | 7305         | 8018    | 12411    |
| SD ration of the area and the convex contour area* | 0.014        | 0.018   | 0.019    |
| SD invariant moment 11*                      | 0.406 \times 10^{-2} | 0.254 \times 10^{-2} | 0.394 \times 10^{-2} |

*SD... standard deviation; p53 negative... p53 negative tumours; IRS 1–4... weakly positive tumours; IRS 5–12... strongly positive tumours; IRS... immunoreactive score.

*Best combination of features with significant difference in the multivariate analysis.
Table 5
Significant different features in p53 positive nuclei of the diploid and tetraploid tumours ($p < 0.05$)

| Feature                                                                 | Diploid | Tetraploid tumours |
|-------------------------------------------------------------------------|---------|--------------------|
| SD skewness of the optical density histogram of the nucleus              | 0.475   | 0.382              |
| in a median filtered image                                              |         |                    |
| Area according line coincidence*                                         | 1735    | 2351               |
| Number of coarse chromatin particles*                                   | 5.256   | 6.424              |
| SD invariant moment 4*                                                   | $0.476 \times 10^{-6}$ | $0.485 \times 10^{-6}$ |
| SD invariant moment 6*                                                   | $0.476 \times 10^{-6}$ | $0.485 \times 10^{-6}$ |

*Best combination of features with significant difference in the multivariate analysis.

Table 6
Significance in different features of tumours with different p53 expression with a S-phase greater than 5% ($p < 0.05$)

| Feature                                                                 | p53 negative | IRS 1–4 | IRS 5–12 |
|-------------------------------------------------------------------------|---------------|---------|---------|
| SD integrated optical density (without correction of section thickness)*| 7699          | 8876    | 14431   |
| SD area according line coincidence                                       | 539           | 707     | 815     |
| Second moment of fine structure                                          | 0.042         | 0.031   | 0.032   |
| SD area of the nucleus                                                   | 574           | 737     | 854     |
| SD convex contour area                                                   | 605           | 776     | 893     |
| SD relative standard deviation of the radial extinction                  | 0.032         | 0.027   | 0.032   |
| SD Standard deviation of the optical density histogram of the nucleus    | 2.804         | 2.128   | 2.601   |

SD... standard deviation; p53 negative... p53 negative tumours; IRS 1–4... weakly positive tumours; IRS 5–12... strongly positive tumours; IRS... immunoreactive score.
*Best combination of features with significant difference in the multivariate analysis.

Table 7
Significance in different features of tumours with different p53 expression with a G2M-fraction greater than 5% ($p < 0.05$)

| Feature                                                                 | p53 negative | IRS 1–4 | IRS 5–12 |
|-------------------------------------------------------------------------|---------------|---------|---------|
| SD area according line coincidence*                                     | 467           | 680     | 778     |
| SD area of the nucleus                                                  | 492           | 719     | 824     |
| SD convex contour area                                                  | 516           | 756     | 864     |
| SD contour difference to a fitted ellipse                               | 0.478         | 0.535   | 0.649   |
| SD integrated optical density                                           | 6864          | 7649    | 12980   |
| (without correction for section thickness)                              |               |         |         |
| SD ratio of the area and the convex contour area                         | 0.014         | 0.018   | 0.019   |
| Contour difference to a fitted ellipse                                  | 1.234         | 1.495   | 1.680   |
| SD number of coarse chromatin particles                                 | 10.270        | 14.950  | 14.950  |
| SD number of nodes in the texture tree                                  | 5.056         | 6.827   | 7.373   |
| SD shape factor of the nucleus                                          | 1.800         | 2.237   | 2.235   |
| SD number of coarse chromatin particles                                 | 5.993         | 6.888   | 7.451   |
| SD “number of nuclei”                                                   | 8.390         | 11.190  | 12.070  |
| SD number of objects darker than 2 × SD of the flat image extinction in | 5.445         | 6.548   | 7.267   |
| a median filtered image                                                 |               |         |         |
| SD integrated optical density                                           | 15773         | 16357   | 28615   |
| (with correction of section thickness)                                  |               |         |         |
| SD invariant moment 3                                                   | $0.210 \times 10^{-3}$ | $0.205 \times 10^{-3}$ | $0.341 \times 10^{-3}$ |
| SD width of the texture tree                                            | 2.583         | 3.261   | 3.471   |
| SD invariant moment 13                                                  | $0.791 \times 10^{-4}$ | $0.566 \times 10^{-4}$ | $0.210 \times 10^{-3}$ |
| SD percentage of heterochromatin optical density and nuclear IOD        | 0.170         | 0.229   | 0.214   |
| Invariant moment 12                                                     | $0.262 \times 10^{-4}$ | $0.226 \times 10^{-4}$ | $0.543 \times 10^{-3}$ |

SD... standard deviation; p53 negative... p53 negative tumours; IRS 1–4... weakly positive tumours; IRS 5–12... strongly positive tumours; IRS... immunoreactive score.
*Best combination of features with significant difference in the multivariate analysis.
Fig. 1. Standard deviation of the percentage of heterochromatin optical density in the nuclear integrated optical density in breast cancers with different p53 expression in correlation to DNA ploidy, S-phase and G2M-fraction. IRS 0 . . . p53 negative tumours; IRS 1–4 . . . weakly p53 positive tumours; IRS 5–12 . . . strongly positive tumours. □ peri-, di- and tetraploid; ■ aneuploid; ○ S-phase < 5%; ● S-phase > 5%; Δ G2M-fraction < 5%; ▲ G2M-fraction > 5%.

As shown in Tables 4–6 and in Figs 1 and 2, the breast cancers with an IRS 1–4 differ from the negative as well as from the more strongly positive tumours (IRS 5–12) in some features of chromatin amount and distribution.

3.2. Comparison of nuclear subpopulations with different p53 expression in groups stratified by their DNA ploidy and their proliferation rate

The nuclear populations differ in their morphology concerning to the p53 expression in the group of peridiploid and peritetraploid tumours and in tumours with a S-phase and G2M fraction higher than 5%, respectively.

The peridiploid and peritetraploid tumours were different in some features of the chromatin distribution and amount. In general, the texture of the nucleus seems to be more homogeneous in the p53 positive population than in the negative nuclei. The mean values of some optical density features were lower in the more strongly p53 positive nuclei. On the other hand, the standard deviation of the integrated optical density increased with the p53 stainability. In the multivariate analysis, the skewness of the optical density was the feature with a significant difference (Table 8; Figs 3, 4).

Breast cancers with a S-phase fraction higher than 5% showed differences in features for the distribution and amount of chromatin, as well. The best discriminant features in the multivariate analyses were the percentage of the heterochromatin optical density of the heterochromatin in the integrated optical density of the whole nucleus and the standard deviation of the integrated optical density.
Fig. 2. Standard deviation of the integrated optical density in breast cancers with different p53 expression in correlation to DNA ploidy, S-phase and G2M-fraction. IRS 0... p53 negative tumours; IRS 1-4... weakly p53 positive tumours; IRS 5-12... strongly positive tumours. □ peri-, di- and tetraploid; ■ aneuploid; ○ S-phase < 5%; ● S-phase > 5%; △ G2M-fraction < 5%; ▲ G2M-fraction > 5%.

Table 8
Significant differences in the nuclear subpopulations with different p53 expression in the euploid (di- and tetraploid) p53 positive tumours ($p < 0.05$)

| Feature                                           | p53− | p53+ | p53++ | p53+++ |
|---------------------------------------------------|------|------|-------|--------|
| Skewness of the optical density histogram of the nucleus' | 0.520 | 0.432 | 0.311 | 0.090  |
| Skewness of the optical density histogram of the nucleus in a median filtered image | 0.456 | 0.373 | 0.258 | 0.0335 |
| Percentage of heterochromatin optical density in the nuclear IOD | 0.366 | 0.401 | 0.438 | 0.537  |
| SD integrated optical density (without correction of section thickness) | 6764  | 8891 | 9762  | 12817  |
| Extinction range of all object thresholds in a median filtered image | 1.859 | 1.798 | 1.728 | 1.579  |
| Mean extinction of the coarse chromatin particles in a median filtered image | 1.290 | 1.272 | 1.260 | 1.234  |
| Mean extinction of the heterochromatin | 1.374 | 1.330 | 1.288 | 1.192  |

SD... standard deviation; p53−... p53 negative nuclei; p53+... weakly p53 positive nuclei; p53+... moderately p53 positive nuclei; p53++... strongly p53 positive nuclei.

'Best combination of features with significant difference in the multivariate analysis.

When the cancers with a high G2M-fraction (higher than 5%) were taken into consideration, many features showed significant differences in the univariate analysis. In these tumours, the irregularity of the nuclear shape increases and correlates with the p53 expression. However, the multivariate analysis resulted in significant differences in two features describing the amount and distribution of chromatin (Table 9; Figs 3, 4).
Fig. 3. Skewness of the optical density histogram of the nucleus in nuclear subpopulations with different p53 expression in correlation to DNA ploidy, S-phase and G2M-fraction. p53−... p53 negative nuclei; p53+... weakly p53 positive nuclei; p53+++... moderately p53 positive nuclei; p53++++... strongly positive nuclei. 

1 peri-, di- and tetraploid; ■ aneuploid; ○ S-phase < 5%; ● S-phase > 5%; △ G2M-fraction < 5%; ▲ G2M-fraction > 5%.

Fig. 4. Standard deviation of integrated optical density in nuclear subpopulations with different p53 expression in correlation to DNA ploidy, S-phase and G2M-fraction. p53−... p53 negative nuclei; p53+... weakly p53 positive nuclei; p53+++... moderately p53 positive nuclei; p53++++... strongly positive nuclei. 

□ peri-, di- and tetraploid; ■ aneuploid; ○ S-phase < 5%; ● S-phase > 5%; △ G2M-fraction < 5%; ▲ G2M-fraction > 5%.
Significant differences in the nuclear subpopulations with different p53 expression in the p53 positive tumours with a S-phase and a G2M-fraction higher than 5% ($p < 0.05$)

| Feature                                      | p53− | p53+ | p53++ | p53+++ |
|----------------------------------------------|------|------|-------|--------|
| S-phase $> 5\%$                              |      |      |       |        |
| SD integrated optical density (without correction of section thickness)$^*$ | 7996 | 9783 | 12143 | 16590  |
| Skewness of the optical density histogram of the nucleus$^*$ | 0.483| 0.405| 0.287 | 0.137  |
| Skewness of the optical density histogram of the nucleus in a median filtered image$^*$ | 0.425| 0.352| 0.234 | 0.080  |
| SD integrated optical density (with correction of section thickness) | 18162| 22103| 25485 | 37368  |
| Extinction range of all object thresholds in a median filtered image | 1.852| 1.799| 1.730 | 1.610  |
| Second moment of fine structure in a median filtered image | 0.242| 0.237| 0.205 | 0.168  |
| Percentage of heterochromatin optical density and nuclear IOD | 0.374| 0.414| 0.450 | 0.520  |
| Mean extinction of the heterochromatin in a median filtered image | 1.369| 1.333| 1.287 | 1.209  |
| Third moment of fine structure | $-0.412$| $-0.492$| $-0.265$| $0.535$ |
| G2M-fraction $> 5\%$                          |      |      |       |        |
| SD integrated optical density (without correction of section thickness)$^*$ | 7123 | 8446 | 1155  | 15715  |
| SD integrated optical density (with correction of section thickness) | 15644| 18204| 24316 | 35378  |
| Skewness of the optical density histogram of the nucleus | 0.499| 0.486| 0.346 | 0.156  |
| Skewness of the optical density histogram of the nucleus in a median filtered image | 0.435| 0.435| 0.239 | 0.100  |
| SD area according line coincidence | 543  | 631  | 734   | 815    |
| Second moment of fine structure in a median filtered image | 0.250| 0.255| 0.218 | 0.174  |
| Extinction range of all object thresholds in a median filtered image$^*$ | 1.834| 1.851| 1.760 | 1.616  |
| Third moment of fine structure in a median filtered image | 0.276| 0.114| 0.379 | 1.025  |
| Contour difference to a fitted ellipse | 1.360| 1.655| 1.661 | 1.658  |
| Third moment of fine structure | $-0.381$| $-0.642$| $-0.413$| $0.396$ |
| Percentage of heterochromatin optical density and nuclear IOD | 0.381| 0.390| 0.428 | 0.515  |
| Shape factor of the convex contour | 13.760| 13.830| 14.060| 14.280 |
| Radius ratio of the nuclear convex contour | 0.719| 0.713| 0.684 | 0.660  |
| Variation coefficient of the contour radii of the nucleus | 0.135| 0.1408| 0.155 | 0.168  |
| SD contour difference to a fitted ellipse | 0.514| 0.568| 0.621 | 0.683  |
| Mean extinction of the heterochromatin in a median filtered image | 1.356| 1.369| 1.306 | 1.212  |
| Extinction range of the lowest possible segmentation threshold | 0.682| 0.644| 0.684 | 0.757  |

SD... standard deviation; p53−... p53 negative nuclei; p53+... weakly p53 positive nuclei; p53++... moderately p53 positive nuclei; p53+++... strongly p53 positive nuclei.

$^*$Best combination of features with significant difference in the multivariate analysis.

4. Discussion

The differences between differently scored tumours were found in tumours with a high proliferation rate and in the groups of euploid and aneuploid breast cancers. The aneuploid cancers as well as the subgroups with a high proliferation rate showed an increase of nuclear polymorphism with the p53 immunoreactive score. Moreover, the aneuploid stronger p53 positive breast cancers also show a higher degree of polychromasia than their p53 negative counterparts. An exception in this part of the study are the peridiploid and peritetraploid tumours which differ only in chromatin distribution features.

The association between the p53 immunostaining and aneuploidy is a well-known fact. A significant relationship between p53 expression and aneuploidy was confirmed in a previous study on breast cancers [11]. Remivikos et al. [27] also found an association between the ploidy state and the p53 expression in cell population in a flow cytometric study of colorectal carcinomas.

Falkmer et al. [9,10] reported different ploidy states in cells of neuroendocrine tumours using a methodological approach similar to the present study.
Other reports describe a correlation between the p53 immunostainability and the S-phase/G2M-fraction in flow cytometric studies [25]. However, we did not find a significant correlation between the p53 expression and the S-phase or G2M fraction in the present study. One possible reason may be the selection of the cases by their p53 status and not by a random or consecutive sampling as in the above mentioned studies.

In all groups with significant differences in the nuclear morphology, the breast cancers with an IRS 1–4 are distinguished from the negative and the more strongly positive (IRS 5–12) tumours in some features describing the chromatin distribution. These findings may suggest a different significance of the immunohistochemically detectable p53 protein [5]. The antibody DO-1 used here recognizes an epitope that is present on both the mutated and on the wild type p53 protein [31]. In another study [18], which includes some cases found in the present one, the p53 gene was found mutated only in tumours with a high IRS percentage of p53 positive cells by means of the PCR analysis. In the tumours with an IRS 1–4/ low percentage of positive cells, the immunohistochemically detected protein may correspond to the wild type of the p53 protein with a prolonged life span. This may be the result of a binding of the p53 protein to other molecules. Another possible interpretation of this phenomenon is an increased expression of the wild type protein caused by a DNA damage. Changes in DNA are a common event in malignomas, but it is not known whether such DNA damage during the malignant transformation and progression may lead to an induction of the p53 wild type expression.

Taking into consideration the different nuclear populations of the p53 positive tumours, it becomes evident that only nuclei from diploid and tetraploid tumours show differences in correlation with the p53 expression in their nuclear features, but not the nuclei of aneuploid tumours. The higher degree of dedifferentiation in aneuploid tumours could be one reason for the missing correlation in p53 expression and the nuclear features in these tumours. This agrees with some findings of a previous study, which examined the morphological nuclear features in correlation with the p53 expression in breast cancer with different stages of the tumour disease, reflected by the clinicopathological features. In this study we found a higher influence of the immunohistochemical detected p53 on the nuclear morphology in cancers in early stage of disease than in those in a more advanced stage [13].

In detail, the nuclei of the peridiploid and peritetraploid tumours with a higher p53 immunoreactivity showed a more homogeneous nuclear texture than their negative counterparts. The mean values for some optical density features decrease with respect to the p53 expression, i.e., the nuclei in the p53 positive populations are paler than the p53 negative cells. In general, the transcriptionally active chromatin is characterized by a lower stainability than the inactive heterochromatin. The paleness of the nuclei in the p53 positive populations may suggest a higher rate of transcription in these cells. The stronger p53 positive nuclear populations showed a greater variability in the chromatin amount than the p53 negative nuclei in these euploid cancers. This observation agrees with the well-known phenomenon of polychromasia. On the other hand, it appears to be contradictory to the non-aneuploid state of these tumours. This group includes tumours with diploid or tetraploid nuclei, as well as tumours with a mixed diploid and tetraploid nuclear population. The chromatin amount reflected by the integrated optical density should also be different in these non-aneuploid tumours.

The whole nuclear population (including the p53 positive and the p53 negative nuclei) of the p53 positive tumours showed a difference between the diploid and the tetraploid cancers only in a feature of the chromatin distribution. In contrast, the p53 positive nuclei of these cases were different in their chromatin distribution as well as in their nuclear size, whereas in the negative nuclear population of the p53 positive cancers no difference could be found in the nuclear features. This may suggest a stronger impact of the p53 positive cell population on the nuclear morphology in both of these ploidy groups.
Significant differences in the nuclear morphology correlated with the p53 expression could be found only in tumours with a high proliferation rate, but not in those with a low proliferation rate. This seems to be in contradiction to the above mentioned differences in the diploid and tetraploid tumour group and the results of a previous study which describes a higher degree of association between the p53 expression and nuclear morphology in breast cancers with favorable prognostic criteria than in those with an unfavorable prognosis. A high rate of proliferation in breast cancers is also a sign of an unfavorable prognosis, but this is not the same as aneuploidy.

The changes in the nuclear populations of tumours with a high proliferation rate with different p53 expression are the same as those in the euploid tumours. Additionally, the p53 positive population in tumours with a G2M-fraction higher than 5% exhibit a more irregular shape than the negative or lower p53 positive nuclei. The loss of p53 function in cells with mutated p53 protein should result in a higher proliferation rate of this cell population. The S-phase and the G2M-phase are also associated with changes in the morphology of the nucleus. The p53 associated changes in nuclear morphology cannot be separated from this proliferation associated nuclear pattern.

In conclusion, the results show the different impact of the p53 state on the one hand, and the DNA ploidy and the proliferation on the nuclear morphology on the other. Furthermore, there are hints for a different impact of subpopulations with a different p53 expression for the nuclear morphology especially in the euploid breast cancers.

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