DNA ploidy and molecular pathological examination of peripheral nerve sheath tumors and synovial sarcomas with special regard to differential diagnostic problems and prognostic factors

Theses

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Introduction

The diagnostics of the soft-tissue tumours as well as the mapping of the molecular differences is one of the most dynamically developing fields of the pathology. Lately, several molecular changes, specific translocations and proteins playing important role in the formation of several types of soft-tissue tumours could be identified successfully. The fusion factors generated by translocations appear as transcription factor and, as they play decisive role in the growth of the cell and the mechanism of differentiation, they influence the development of the soft-tissue tumours as well. The understanding of the molecular mechanisms could help significantly in the designing of the therapy or in some cases the specific, so called selective therapies; a good example for that is the very successful Glivec treatment of the Gastrointestinal Stromal Tumours (GIST).

To our today's knowledge, the soft-tissue sarcomas can be divided into two groups by the complexity of their molecular differences. To the first group belong the sarcomas with special translocation, fusion factor and protein mentioned above having simple cytogenetical differences (synovial sarcoma, myxoid liposarcoma etc).

These types of tumours are formed "de novo" and the formation of the fusion factor must necessarily be the initial step in the growth of the tumour. The other group of the sarcomas (e.g. MPNST, leiomyosarcoma) is constituted by the tumours of complex karyotype without any fusion factors. These tumours are obviously genetically instable. They can be formed "de novo" as well, but they happen not so rarely that they develop from a previous „dysplastic-precursor" lesion similarly to the epithelium tumours.

Our study presents both groups. While for the case of the peripheral nerve-sheath tumours the „dysplastic-precursor" process was emphasised, for the synovial sarcomas, representing the other group, the examination of the prognostic factors and the possible options of the therapy was preferred.

Defining the problem, goals

1. Schwannomas (sh-won-oma-s). There are several sub-classes of Schwannomas that may raise serious differential-diagnostic problems.

Ancient schwannoma

The lasting Schwannomas show frequently extended stromal and vascular degenerative deformations like bleeding, large-scale hyalinisation and certification. The nuclei of the cells of such tumours show progressive degenerative deformations, like pleimorphism of the nucleus, hyperchromasia or pseudoinclusions appearing in the cytoplasm. This type of the tumours is called “ancient Schwannoma”. It cannot be distinguished clinically from the conventional Schwannomas.

Neuroblastoma-like Schwannoma

Instead of the classical elongated Schwann cells, the tumour is composed of small round tumour cells (resembling the neuroblastoma) arranged in rosette formation. There may appear mitotic figures, but in low numbers only. The “giant rosettes” are essentially modified Verocay bodies.

Cellular Schwannoma

As indicated by its name, the cellular Schwannoma shows higher than average cell density, the number of mitoses is higher and the Verocay bodies are visible. Under such circumstances the discrimination from the well differentiated MPNST may not be easy.

Comparing these types to the conventional Schwannomas and neurofibromas, the objective criteria may help in the correct diagnosis. Such an objective criterion can be the examination of the chromosomal

instability. The literature data are still contradictory in that respect that the benign Schwannomas are euploid or there are aneuploid benign tumours as well. Our goal was to see whether the benign Schwannomas showed euploid-poliploid properties or by a deeper analysis – FISH analysis – it was able to observe eusomic-polisomic properties.
Our goal is to facilitate the setting up of right diagnosis by these two examinations and gain insight into the mechanism of the malignant transformations of such tumours.

Synovial sarcoma

Analysis of the prognosis of synovial sarcoma – contrary to the earlier beliefs according to which the prognosis is very poor – shows that it is in fact strongly variable and depends on several prognostic factors. Our goal was the examination of the prognostic value of the ploidity and the determination of the Her-2 amplification status and its possible diagnostic significance.

Material and methods

Peripheral nerve-sheath tumours

The 44 peripheral nerve-sheath tumours were taken from the Department for Oncopathology of the Semmelweis University (Szent János Hospital of the City Council).
27 benign Schwannomas were chosen containing 5 ancient, 2 cellulars (pseudosarcomatosus), 2 neuroblastoma-like, and 1 multiplex version.
The 9 neurofibromas contained one atypical-cellular sub-type.
Of the 8 malignant peripheral nerve-sheath tumours 2 tumours were of malignant epitheloid variant.
The follow-up period was at least 5 years.

The histology, nucleus isolation and the DNA measurement were carried out at Ist Department of Pathology and Experimental Cancer Research, while the FISH analysis was combined with an automatic image analyse system (Metafer 4) and took place in the Institute of Pathology at Bonn (Germany).

Synovial sarcoma

Samples taken from synovial sarcoma fixed in formalin and embedded in paraffin originating from the Department of Pathology of “Szent János” Hospital and National Institute of Oncology were used. For the studies primary tumours were always available. The tumour samples stored at -80 C° were available in every case for the application of the fluorescent in situ technology.

Immunohystochemistry (peripheral nerve-sheath tumours)

Monoclonal antibodies were used for the determination of the immune-phenotype (S-100 protein) confirming the correct diagnosis in the more problematic cases, as e. g. in case of the ancient, cellular, and neuroblastoma-like Schwannomas. Further anti-bodies are: alpha-smooth muscle-actin, desmin, Ki-67, CD99 (Novocastra UK).
In all cases the solutions suggested by the protocol were used. The procedure was made with a Vectastain (Novocastra UK) kit and 3-amino 9-ethylcarbasol was used as markers. In all cases both negative and positive controls were used.

Interphase cytogenetics (FISH analysis, peripheral nerve-sheath tumours)

For the purpose of the interphase cytogenetic analyses a nucleus isolation smear was taken of smears originating from aspiration cytology. The nucleus isolation smears were made of a paraffin block. Sections of 50 μ

thickness were cut from the paraffin block followed by a conventional re-hydration process. Then, the sections were digested in 2ml pepsin of 0.5 % concentration at pH 1.5, at 37 C° for 60 minutes. The digestion was controlled by microscope. The process of the digesting was stopped by a cold phosphate buffer (PBS). The nucleus isolation suspension was filtered through a nylon foil and the deposit was separated. The deposit was re-suspended by a citrate buffer of 0.01% and the residue was cytospinned, the supernatant material was separated and put onto a slide.

3 FISH tests were used, the 7, 17a and 18 specific centromeric probes characterising the chromosome: (alpha7 and 17 CEP Spectrum Green, alpha18 CEP Spectrum Orange, Vysis Inc., USA). The 17 and 18 chromosome samples were applied in pairs.
In the course of the double marking, 5 μl of hybridising solution was prepared containing 10 μg of probe and 60% of formamide with 2X sodium saline citrate (SSC) and the cell preparation was covered with a plastic plate. The samples were denaturated simultaneously at 82 C° for 4 minutes followed by a hybridisation at 37 C° in a wet chamber for one night. The washing following the hybridisation was carried out as follows: washing for 3x5 minutes in formamide/2XSSC of 60% concentration at pH 7, at 37 C°, then washing for 3x5 minutes at room temperature in 2x SSC. For the covering, Vectashield material was applied. This material contained 0.02 μg DAPI (4-6 diamino 2 phenilindol). The DAPI stains the nucleus blue, while the Vectashield contains a material preventing the fading; then, the sample was covered with a glass plate.

Automatic image analysing system of the FISH signals

The image analyser is able to take up 8 glass slide holder simultaneously. The image analysing system: Meteafer 4- Meta Cyte (Meta Systems Germany) being able to measure the set quantity of cells (300) for each glass slide holder by a software, fix the images of the individual cells, group

and query the images taken. The signals on the images can be counted and, when clicking on the image, the system automatically re-localises the image, i. e. the results can visually be interpreted. The system counts automatically the signals present.

Immun-histochemistry (synovial sarcoma)

The tissue samples fixed in formalin and embedded in paraffin were de-paraffinated and re-hydrated. Her-2 polyclonal rabbit antibody was used with the En vision+ system (Dako, Carpenteria, CA). The antibody (catalog number AO485) was diluted at 1:2000 and used at room temperature for 60 minutes.
The section was pre-treated in EDTA with microwave antigen retrieval at pH 8.
The solution was incubated with endogen peroxydase at room temperature for 10 minutes. The breast carcinoma showing 3+ Her 2 positivity was used as positive control, while the case when the primary anti-body was left out from the parallel staining, was used as negative reference.
The immune-reactivity of the cytoplasm and the plasma membrane was always analysed.
The Her-2 positivity was evaluated by standard methods in the way as follows:

0: no plasma membrane staining

1+: cytoplasma and/or weak partial plasma membrane staining

2+: weak full plasma membrane staining in more than 10% of the cells.

3+: strong full menmbran staining in more than 10% of the cells.

Because in synovial sarcoma the Her-2 positivity is less intensive as compared to breast carcinoma, we considered the cytoplasmic positivity as 1+ positivity.

Inter-phase fluorescent in situ hybridisation for the indication of the Her-2 amplification (synovial sarcoma)

For the fluorescent in situ hybridisation of the Her-2 gene a smear taken from a tumour bank was used.
The dual-colour chromosome technique was used, 17q12 (Her-2/neu) / alpha satellita 17 chromosome mix was applied with directly marked probe (Q biogene, France).
The hybridisation solution of 5 μl volume contained 5 μg probe, 60% formamid with 2x sodium saline citrate (SSC). This solution was dropped to the cell material and the system was covered with a plastic plate. The samples and the probes were denatured at 75 °C for 5 minutes, then, the hybridisation at 37 °C took place for one night. The post-hybridisation washing took place as follows: washing in 60% formamid with 2X sodium saline citrate at pH 7, at 37 °C for three times 5 minutes and washing in 2x sodium saline citrate (SSC) at room temperature at 3 x 5 minutes. The preparation was put onto a slide, covered with Vectashield material containing 0.02 μg of DAPI-t (4-6 diamino 2 phenilindol) for the purpose of nuclear staining. (Vector Laboratories, Burlingame, CA). In all cases the recommended quantities and dilutions were used. The evaluation of the slides was performed using a double- and triple-filter microscope (Olympus BX 40.).
100 randomly selected tumour cell nuclei were evaluated for each smear in combination with an image analysing system. According to the definition, a case was qualified as amplified, if the number of the Her-2 signals divided by the number of the 17 chromosome signals resulted in a figure above 2.

Determining the DNA content with image analyser (peripheral nerve-sheath tumours and synovial sarcoma)

The nuclear DNA content was determined by applying the adsorption cyto-photometric DNA testing method. The smears were fixed in formaline of 4% concentration for 30 minutes. The samples were stained with Schiff reagent

using the Feulgen stoichiometric method (Merck, Darmstadt, Germany), in compliance with the ESAP consensus. The DNA image analysis was made with a DNA measurement software applied to the microscope (CYDOK R, Fa., Hilgers, Königswinter, Germany). This system fulfils all requirements needed to the implementation of an accurate DNA adsorption cyto-photometry.

No. 40 objective and interference filter were used (565 +/-10nm). The DNA content of at least 100 diagnostic cells was determined for every smear (this number was 200-300 in several cases). For the determination of the optical density by the Feulgen staining, the DNA content of reference cells (lymphocytes and granulocytes) was determined. The DNA content of these cells, as the DNA content of normal diploid cells (2c) was used as a standard. The variation coefficient of the reference cells fell in the range 3 - 5 %. The reference cells were non-tumour cells in the smear. Finally, the DNA content of 30 reference cells and 100 tumour cells was determined and in the end of the measurements, the computer plotted a histogram using image analysis method. For the definition of the aneuploidity the „stem line" interpretation was used by Haroske.

The determination of the DNA index takes place based on the classical aneuploidity determination, the DNA content of the measured cells will be divided by modal DNA content of the measured cells. A tumour is dipoid, if this ratio is 1+/- 10%, and it is aneuploid, if this ration is below or above 1+/- 10%.

Statistical method

For the statistical analyses the chi-square test was used; the difference was considered significant, if $p < 0.05$.

Results

Peripheral nerve-sheath tumours

Adsorption cyto-photometric DNA test

44 peripheral nerve-sheath tumours' ploidity examinations were carried out. All benign Schwannoma, including the cases problematic in terms of differential-diagnostics, were diploid. 25 of 27 showed characteristically euploid-polyploidisation (92.5 %). The 4c peak value exceeding 10% was observed in 20 cases of 27 (74%). All neurofibromas (9 cases) including the atypical sub-types as well, proved to be diploid and 2 cases of them showed euploid-polyploidisation (22.9 %), but the 4c peak value did not exceed 10%.

3 of the 8 malignant peripheral nerve-sheath tumours proved to be diploid, while the remaining 5 were aneuploid. These tumours did not show the signs of euploid-polyploidisation.

In 1 one case of 8, the 4c peak value was higher than 10% but the 4c region of these cells is constituted factually of G2 phase cells, because these cells are real tetraploid- tetrasomic cells, verified by FISH analysis as well.

The S phase fraction was not specified, as the 100 cells measured were too few in number.

Inter-phase cyto-genetics, FISH analysis

The counted signals and signs can be divided into two groups, eusomic – poliosomic signs; number of the signs: 2, 4, 8, 16, and the aneusomic signs, number of signs: 1, 3, 5, 6, 7.

The cells for which the analysis of the signals was impossible even after a relocation, were left out of the evaluation. The average of the inadequate cells was 4.1%, while the interval was 1.3-7%. Polysomia was observed in the benign Schwannomas.

The average value was 13.9%. This result harmonises with the findings of the adsorption cyto-photometric DNA test.

The polisomia is significantly higher in cases of ancient Schwannoma (30.1 %), harmonising with the finding that the tumour cells contain many enlarged, hyperchromatic and multi-lobulated nuclei at this type of Schwannomas. The polysomia was observed at the atypical neurofibroma as well (4.3%), but no polisomia was found in the malignant peripheral nerve-sheath tumours. Tetrasomia was found in the peripheral nerve-sheath tumour (case No. 44, 0.7%). In the same tumour, the 4c peak value exceeded 10%. The cells showing the 4c peak value were really G2 phase cells (containing double DNA quantity) and not authentic tetrasomic cells. The centromeric regions of the chromosome pairs of such G2 phase cells are connected, because the chromosomes do not separate during the mitosis and in the tests centromeric DNA probes were used giving normally 2 signals for every chromosome. In case of an authentic tetrasomia, the chromosomes would provide 4 signals.

Very interesting is the observation that a low percentage of the benign Schwannoma showed aneusomia; the share of the aneusomic cells was 2.58% as compared to the total number, while the interval was 1.33-3.44.

On the contrary, the atypical neurofibroma showed marked aneusomia (18.44 %), but 4.3%- of the neurofibromas presented polisomia as well. It is important to note that if the monosomia and trisomia are equal in number, the aneusomia is called „balanced", aneusomia relating to the total DNA content and in this case the DNA is index diploid. Two diploid chromosomes (89.6% , case No. 44, trisomia), the other one is the chromosome No. 18 (91.6%, case 40, monosomia).

The cases (5 benign cases in total) for which 300 cells could not be measured were left out of the analyses (for technical reasons), although these tumours showed similar results as the measured 6 benign Schwannomas.

Synovial sarcoma

Clinical-pathologic data

The age of the patients was between 13 and 68 years with an average of 39.8 years. The size of the tumour exceeded 5 cm in all cases. The follow-up period ranged from 16 to 78 months (the average follow-up period was 48.5 months).
All patients were treated with Doxorubicin mono-therapy (75mg/m^2 on the first day, repeated for 21 days for 6 cycles). In 10 cases local recurrence developed, in 9 cases lung metastasis was found, while in 4 cases both recurrence and lung metastasis were observed. 3 patients died as the result of the tumorous process.

Histological diagnosis

Besides the characteristic histological picture (bi- or mono-phasic), the diagnostic of tumours took place by immuno-histochemical auxiliary methods and, if it was required, by molecular supportive method as well; in the latter case a characteristic t(X,18) was indicated.
Besides the vimentin positivity, the tumours showed at least focal cytokeratine and/or EMA positivity in the monophasic cases, while in the biphasic cases the glandular or obviously epithelial component showed naturally strong cytokeratine and/or EMA pisitivity. The S-100 reaction showed in individual cases focal positivity, but consequent negative reaction was found with the alpha smooth muscle actin, H-Caldesmon, CD-34, CD-31, Leu7 and CD-117 reactions. If the epithelal marker positivity was present only focally, the translocation FISH reactions were carried out with X painting and 18 centromeric probes; the reactions proved to be positive in all cases.

Indication of the Her-2 protein in synovial sarcoma by immuno-histochemical examination

In 4 cases of 20, weak focal cytoplasm positivity (1+) , in 3 cases of 20 strong positivity was observed in both the plasmamembrane and the cytoplasm (2+). Strong positivity (3+) was not observed.
Concerning the quality of the painting, no difference was observed between the epitheloid and spindle cell areas. Totally, the 7 positive cases have represented 35% of the synovial sarcomas.

Examination of the Her-2 gene amplification in synovial sarcoma by fluorescent in situ hybridisation.

Carrying out the FISH analysis, amplification of the Her-2 gene (15%) was found in 3 cases.
The rate of amplification was 2-3-fold in all cases, i. e. in more than 10% of the cells evaluated. No massive amplification was observed.
The Her-2 oncogene amplified cases were the same as for which 2+ positivity was found by immune-histochemistry. In the cases of 1+ positivity no amplification was observed.
In one case, the trisomia of the 17 chromosome was found, while in another case polisomia of the 17 chromosome was observed, but these facts did not influence the expression status of the Her-2 protein.

DNS cyto-metrics

6 aneuploid, two biphasic and 4 mono-phasic cases of 20 synovial sarcomas were found (30 %). Of the aneuploid cases one was hypodiploid; in this case the DNA index was 0.89. Furthermore, 11 mono-phasic and 3 bi-phasic cases were diploid. One of the cases showing 3 Her-2 gene amplification was aneuploid, while the other two proved to be diploidnak.

Correlation between the Her-2 status, DNA ploidity and prognosis.

The amplification of the Her-2 gene showed significant correlation with the lower risk of metastases ($p < 0.05$).
None of the 3 amplified cases gave metastases; this means significant correlation, but no significant correlation was found between the recidiva building and the amplification itself.
Furthermore, no correlation was found between the status of the Her-2 gene and ploidity. Again, no correlation was found between the ploidity status, formation of metastases and recidiva building.

Main statements and conclusions of the study

Euploid-poliploidisation characterising the benign Schwannomas – including the sub-types causing diffential-diagnostical problems – were found and the 4c peak value is generally higher than 10% of the total number of the cells measured. This phenomenon is not characteristic to the neurofibromas and MPNST.
Our results are contrary to the idea suggested by the literature that one part of the Schwannomas would be aneuploid. It was pointed out that this idea was generally based on the misinterpretation of the euploid-poliploidisation and confirmed that the benign Schwannomas are in fact diploides.

We gave an account among the first authors of the balanced aneusomia shown in low percentages of the benign Schwannomas and the fact that it occurs more frequently in the atypical peripheral nerve-sheath tumours, meaning that the tumour qualifies as a „ dysplastic-precursor" lesion.

To our best knowledge, we are the first to identify the Her-2 gene amplification in the synovial sarcoma by the FISH method.

For synovial sarcomas significant correlation was found between the Her-2 amplification and longer survival, but there was no positive correlation between the amplification and chances of recurrences.

Our own findings did not confirm that ploidity was an independent prognostic factor in the case of synovial sarcomas.

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