Short thesis for the degree of doctor of philosophy (PhD)

Studying biological mechanisms leading to chromosome number instability in malignant tumours

by Katalin Hegyi

Supervisor: Gábor Méhes MD, PhD

University of Debrecen

Doctoral School of Clinical Medicine

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Supervisor: Gábor Méhes MD, PhD

Doctoral School of Clinical Medicine, Doctoral Programme of Experimental and Clinical Oncology University of Debrecen

Head of the Examination Committee:	Klára Matesz MD, PhD, DSc
Members of the Examination Committee:	György Panyi MD, PhD, DSc László Torday MD, PhD

The Examination takes place at the Library of Department of Pathology, University of Debrecen, Faculty of Medicine, on September 10th 2014, at 11:00.

Head of the Defense Committee:	Klára Matesz MD, PhD, DSc
Reviewers:	Csongor Kiss MD, PhD, DSc Tibor Krenács PhD
Members of the Defense Committee:	György Panyi MD, PhD, DSc László Torday MD, PhD

The PhD Defense takes place at the Lecture Hall of Building A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, on September 10th 2014, at 13:00.

INTRODUCTION

Increased cell proliferation rate resulting in cell proliferation errors is the most prominent hallmark of malignant transformation. The repair mechanisms are damaged, the metabolic profiles of tumour cells are changing. If this process results selection advantage of tumour cells, it leads to the enrichment of malignant cells.

Chromosome segregation is tightly regulated, the malfunction of the process results aneusomy and aneuploidy, thus changed gene expression, gene product function and diverse gene dose based effects.

Quantitative changes in DNA content manifest at morphological level as well marked by irregular, often bizarre nuclear morphology. Chromosome number changes are often correlate with the malignant capacity of a given tumour type. Mutations lead to accumulation of genetic alterations during carcinogenesis.

The proper centrosome cycle and mitotic checkpoint are essential in the regulation of chromosome segregation and equal chromosome distribution between daughter cells.

Recent experiments revealed that Aurora kinases are key regulators in the above mentioned processes, thus in ensuring genetic stability during cell division. Expression changes in these kinases contribute to genetic instability. The overexpression of the kinase family, in particularly of Aurora A and B is reported in various human tumours.

The important regulatory role of Aurora B on ensuring normal ploidy is known: in early G2 phase the kinase initiates chromosome condensation by the phosphorylation of histone H3. Furthermore as a member of CPC (Chromosome Passenger Complex) it regulates the chromosome-mitotic spindle interactions.

Under experimental conditions the inhibition of Aurora B kinase function leads to significant decrease of histone H3 phosphorylation, thus genetic instability and appearance of aneuploid cell populations and apoptosis.

On the other hand, the overexpression of the kinase results increased histone H3 phosphorylation and defective chromatin condensation in HeLa cells.

The multiple regulatory role of Aurora B in mitosis and the kinase's upregulation is well known in several tumour types, therefore the kinase is a promising target of anti-tumour drugs.

Determining adequate targets for Aurora kinase inhibitor therapies are still controversial: mainly based on fact that the kinase is overexpressed in many types of malignancies. The kinase expression is studied alone as an independent biomarker, other potential interfering factors are usually disregarded.

There is no consensus in defining Aurora B overexpression: it is studied by different methods at mRNA or protein level.

Our experiments focused on the clarification of the possible correlation between changed cell proliferation rate and Aurora B kinase function in different tumour tissues. Our data could help in the selection of adequate anticancer therapy by the biological classification of malignant disease, tumor grading and prognosis.

By examining Aurora kinase B and its targets it may be possible to identify predictive markers which are applicable in routine histological and laboratory diagnostics.

Taken together, our main goal was to identify a cellular biomarker which may indicate cell division defects and which prognosticate the possible effects of anti-mitotic drugs.

Our experiments mainly focused on the effects of Aurora B kinase on chromosome number and chromatin structure, the correlation between changed kinase expression and formation on cell populations showing aneuploidy.

MAJOR AIMS

Our experiments focused on the clarification of the possible correlation between Aurora B kinase function and aneuploidy in malignancies.

We carried out our experiments on Aurora B and interacting proteins immunohistochemistry on invasive breast carcinoma and aggressive B-cell lymphoma samples.

The copy number changes of chromosome 17 and TP53 and URKB containing 17p13.1 region were studied by fluorescence in situ hybridization method. Correlation between chromosome 17 copy number and ploidy was assessed by flow cytometry.

Our main goal was to identify a cellular biomarker which may indicate cell division defects and which prognosticate the possible effects of anti-mitotic drugs.

Aims of experimental study:

1. Studying Aurora B expression on invasive breast cancer and aggressive B-cell lymphoma samples using immunohistochemistry

1.1. Defining Aurora B overexpression under tissue conditions

1.2. Examining the correlation between Aurora B expression levels and totel cell proliferation activity

1.3. Studying the main Aurora B upstream és downstream interacting proteins' expression

2. FISH analysis of AURKB, TP53 and chromosome 17 on invasive breast cancer and lymphoma samples

2.1. Studying the possible cytogenetic background of Aurorra B overexpression

2.2. Studying TP53 and AURKB containing 17p13.1 locus status

2.3. Determination of chromosome 17 copy number changes and ploidy by FISH and flow cytometry

3. Studying the possible connections of Aurora B overexpression and aneusomy

4. Collection of clinical correlations, comparative analysis with laboratory findings

MATERIALS AND METHODS

Invasive breast cancer samples

Histological samples of fifty patients diagnosed with invasive ductal and lobular breast carcinoma were recruited to the study. Samples were fixed in formalin for 24h and processed routinely into paraffin blocks.

Relevant normal control specimens (normal, surgically resected breast tissue) were not available.

45 tumour samples were showing invasive ductal carcinoma morphology and the remaining five cases were invasive lobular carcinomas. ER and PR positivity was observed in 36 and in 27 cases, respectively. 16 cases were c-erb-B2 expression negative. We have observed HER2 gene amplification in 16 cases.

Lymphoid tissue samples

Fifty patients with aggressive B-cell lymphoma were recruited to the study diagnosed between 2004 and 2012 in the Department of Pathology, University of Debrecen. Specimens consisted of surgically resected lymph node samples.

Following the histopathological evaluation according to the WHO 2008 classification 43 cases were defined as DLBCL, not otherwise specified (NOS); 3 cases as primary mediastinal large B-cell lymphoma (PMBL), 3 cases as ALK+ large B-cell lymphoma (B-ALCL) and 1 case as B-cell lymphoma, unclassifiable, with features intermediate between diffuse large B-cell lymphoma and classical Hodgkin lymphoma. DLBCL, NOS cases were subclassified on the basis of cell origin according to Hans into germinal center B-cell–like (GCB) and non-GCB subclasses by the expression of CD10, Bcl-6, and MUM-1 determined following immunohistochemistry.

Tissue microarray blocks were prepared for archiving and IHC data confirmation purposes.

10 reactive lymph node samples showing florid follicular hyperplasia were used as non-neoplastic control. Both of the control and lymphoma samples were fixed in 4% formaldehyde in PBS for 24h and embedded into paraffin blocks in a routine process.

Tissue microarray

Tissue microarray blocks were prepared for archiving and IHC data confirmation purposes.

After labeling the representative tumor areas on hematoxylin-eosin (HE) stained slides, 3-3 tissue cores 1 mm in diameter were removed from donor FFPE blocks by using TMA Master (3DHistech Kft, Hungary) and replaced to a recipient block. Normal human liver tissue cores were used for orientation. Tissue core coordinates were digitalized with TMA Master 1.0 software (3DHistech Kft, Hungary).

The gaps between tumor tissue cores and recipient block were filled with melted (56 °C) paraffin.

Immunohistochemistry (IHC)

By IHC method the total proliferating cell fraction /mouse anti-Ki67, MIB-1 clone/, the Aurora B /rabbit anti-Aurora B/, survivin (mouse anti-survivin/ and phosphorylated histone H3 (H3S10P, rabbit anti-histone H3, phospho-Ser10) expressing cell fractions were detected on FFPE samples.

Immunohistochemical labeling was carried out on 3 µm thick sections on adhesion slides (SuperFrost Ultra Plus®, Menzel-Gläser, Germany) according to manufacturers' instructions. Briefly: after deparaffinizing (xylene, 2×3 min) sections were rehydrated (ascending ethanol series, 3-3 min) than epitopes were retrieved (high pressure cooker, 3 min). After washing steps (1xTBS, 2×5 min) and blocking (1% BSA, 0.1% NP-40 in 1xTBS 120 min) primary antibodies were applied. After 2 hours incubation, the non-binding antibodies were washd out (1×TBS), and sections were incubated with secunder antibodies for 40 minutes. After washing steps (1xTBS, 2×5 min), the antigen-antibody bindings were visualized with DAB reaction (0.02 v/v% DAB in DAB buffer). After washing steps slides were counterstained with hematoxylin (Gill II hematoxylin, 5 sec). After dehydration in acetone and xylene slides were mounted with xylene-based medium (Surgipath Micromount, Leica Microsystems, Germany).

Evaluation was carried out with Leica DM 2500 transmitted light microscope according to *Trasmitted light microscopy* chapter.

Trasmitted light microscopy

Ki67 (MIB-1), Aurora B and survivin expressing cells were determined in the percentage of total tumour cell fraction.

H3S10P positive cells were counted in 10 high power fields (/10 HPF).

To enable appropriate comparison with physiological B-cell proliferations reactive germinal centers in 10 control lymph node samples were selected and evaluated by the same criteria.

Aurora B expression relative to the total cell proliferation activity was given as the Aurora B/MIB-1 index (referred as AMI). Overexpression of Aurora B was defined at AMI values higher than 0.5, that was calculated on the basis of data obtained from the control samples (mean AMI+2×SD).

In invasive breast carcinoma samples appropriate control specimens were not available. The cut off value for Aurora B overexpression was set at 0.3 on the basis of previously published data.

Mitosis index was counted on haematoxylin and eosin stained tissue sections in 10 high-power fields.

Fluorescence in situ hybridisation (FISH)

Chromosome 17 and TP53 copy number was determined with direct labeling method, using the LSI TP53/CEP17 probe (Abbott Laboratories Inc., Downers Grove, IL, USA). AURKB copy number was evaluated using the PoseidonTM Repeat FreeTM AURKB/SE17 (Kreatech Diagnostics, Amsterdam, The Netherlands) probe.

Deparaffinized tissue sections were pretreated according to the manufacturer's instructions.

Briefly: 5 μm thich sections were Deparaffinized in NeoClear (Merck, Germany, 3×10 min), than in case of Abbott DNA probes were incubated with 0.2 M for 20 min. After these steps slides were incubated with 0.8 M NaSCN (20 min, 80°C) and 0.4 m/V% pepsin solution (10 min, 37°C). After pretreatment 5-5 μl of hybridization mix containing DNA probes were applied. Codenaturation with TP53/CEP17 probe was carried out on 75°C for 10 min, with AURKB/SE17 probe on 80°C 5 min. Hybridisation was carried out on 37°C overnight. Codenaturation and hybridization steps were done by

using ThermoBrite (Abbott Molecular) semi-automatized hybridization chamber.

Non-hibridised DNA probes were washed out with detergent containing buffers (0.3% Triton X-100/2xSSC).

Nuclei were counterstained with DAPI (1000 ng/ml, Abbott Molecular or Kreatech Diagnostics).

Determination of HER2 gene status were carried out by using DAKO HER2 FISH PharmDx (DAKO, Denmark) kit. Results were reproduced by using DAKO HER2 Instant Quality FISH PharmDx (DAKO, Denmark) kit.

In situ hybridization was performed on 5 µm thick sections with both HER2 FISH pharmDx™ (DAKO) and HER2 IQFISH pharmDx™ (DAKO) probe kits on serial sections according to the manufacturer's protocol.

Deparaffinised sections (NeoClear, Merck, Darmstadt) were pretreated with MES (2-[N-morpholino]ethanesulphonic acid, provided by the two kits) buffer followed by proteolytic digestion using pepsin (provided by the kits). Ready-to-use pepsin solution was dropped on slides for performing conventional FISH, and diluted pepsin solution was applied in cuvette for IQFISH. After washing in 1xwash buffer (provided by the kits) slides were dehydrated through a graded series of ethanol and the ready-to-use probe mix was applied to the sections, coverslipped and sealed.

Denaturation and hybridization were performed in a hybridization chamber (StatSpin ThermoBrite, Abbott Molecular). Slides were denatured at 82°C 5 minutes for conventional HER2 pharmDx™, and at 66°C for 10 minutes for the IQFISH probe. Hybridization was performed overnight for the conventional HER2 FISH and 90 minutes for HER2 IQFISH protocol at 45°C in both options. Stringent wash was performed at 65°C for conventional FISH and at 63°C for IQFISH 10 minutes for both kits. Slides were dehydrated and covered with DAPI containing antifade solution and coverglass. Storage was at 4°C in the dark until evaluation.

FISH signals were detected with Zeiss Axio Imager Z2 fluorescence microscope and the Isis imaging system (MetaSystems, Altlussheim, Germany) for image acquisition according to the following *Fluorescence microscopy* chapter.

Fluorescence microscopy

FISH signals were detected with Zeiss Axio Imager Z2 fluorescence microscope and the Isis imaging system (MetaSystems, Altlussheim, Germany) for image acquisition.

In invasive breast cancer samples FISH signals were counted in 100 nuclei in each case with a 63x objective.

Chromosome 17 status were determined as follows:

a) disomy: on the basis of follicular hyperplasia samples the cutoff was set at 1.814 +2×SD (0.081)=1.98 signals per nucleus
b) hyperdisomy: between 1.98 and 2.3 signals per nucleus
c) trisomy: 2.3-3.3 chromosome 17 copies per nucleus
d) tetrasomy: 3.3-4.3 signals per nucleus

AURKB and TP53 deletion was defined at copy numbers ≤0.8 per chromosome 17 based on earlier measurements on normal tissue sections.

Determining DNA content by flow cytometry (ploidy tests)

Ploidy tests of B-cell lymphoma and follicular hyperplasia samples were carried out on isolated cell nuclei suspensions in UD MHSC, Department of Biophysics and Cell Biology.

Isulated nuclei were prepared from FFPE samples according to Hedley with slight modifications. 50 μm sections were prepared from FFPE blocks. After deparaffinization (NeoClear 3×10 min) sections were incubated in 0.8 m/V% pepsin solution (120 min at 37 °C, vortexing in every 10 minutes). After pepsin treatment suspensions were filtered (nylon filter with pore size 50 μm). After centrifuging filtered suspensions (3000 g, 8 min) nuclei were resuspended in 1 μg/ml DAPI in 1xPBS solution.

DNA content could not be measured on invasive breast carcinoma samples, nuclei isolation was not successful.

DNA content was measured by using FACSAria II flow cytometer and FACSDiva v6.1.3 software. 100.000 events were recorded for each sample.

DNA index were determined with FCS Express 4 v. 4.07.0005 (DeNovo Software, USA) software.

Nuclei suspension isolated from FFPE reactive lymph node samples were used as controls.

Statistical analysis

MedCalc (version 12.2 for Windows, MedCalc Software, Mariakerke, Belgium) SPSS (version 20.0, IBM Corporation, Armonk, NY, USA) and MS Excel XLStat (v7.5.2, Addinsoft, USA) statistical softwares were used for statistical analysis. Mean and standard deviations were calculated for each group and analyzed with Student's t-test. P values <0.05 were considered statistically significant.

Correlations between datasets were obtained using linear regression analysis. Linear correlation was stated at correlation coefficient (R) higher than 0.7.

Overall survival probability was calculated by Kaplan-Meier method.

Significancy between survival rates were calculated using log-rank test. P values <0.05 were considered statistically significant.

RESULTS

Aurora kinase B expression on invasive breast carcinoma samples

The rate of Aurora B expressing cells in the studied breast carcinoma cases was found in the range of 1-35% (mean=6.15, SD:8.8) with a subset of breast carcinomas showing highly elevated Aurora-B expression. The cell proliferation capacity expressed by the MIB-1 index was in the range of 1-95 (mean=19.2, SD:20.7) in the same tumors.

Breast carcinomas with HER2 amplification presented with an elevated Aurora B fraction (p=0.02) however these samples also showed significantly higher MIB-1 proliferation index (p=0.018).

Triple negative (HER2-, PR-, ER-) samples showed also a significant increase in Aurora B expression (p= 0.001), an elevated cell proliferation was seen as well (p= 0.0003).

A strong correlation between Aurora B protein expression and total cell proliferation rate (regression coefficient=0.77) was shown.

To obtain a more accurate picture on Aurora B expression relative to the cell cycle in proliferating cell populations the ratio of the Aurora B and the MIB-1 expressing cell population (Aurora B/MIB-1 index, AMI) was calculated which was found in the range of 0-1 (mean=0.32, SD:0.28).

According to observations on highly proliferating normal cell lines and reactive changes the group of low and high Aurora B expressing tumors was determined by using an AMI cutoff set to 0.3. 20/50 (40%) of the studied breast carcinomas showed an AMI higher than 0.3 and thus were considered as Aurora B overexpressing.

The AMI was found to be independent of the HER2 status.

HER2 gene status determined by HER2 PharmDx and HER2 IQFISH PharmDx kit

We found a 100 % accordance regarding HER2 amplification and chromosome 17 copy number between the new one-day and the conventional FISH tests indicating the absence of bias between the two methods. The correlation between the HER2/CEN17 ratios was very good.

Reaction interpretation problems were virtually absent in case of the one-day approach in the evaluated series of cases but a high fluorescence background interfered with the FISH signal in three samples resulting ambiguous values by the conventional HER2 pharmDX kit.

The use of the IQFISH approach eliminated most of the autofluorescence and tissue damage was also much less obvious in any of the slides.

Copy number alterations of the loci 17p13.1 and of chromosome 17

For examining the possible cytogenetic background of Aurora B overexpression FISH analysis of the samples was carried out.

FISH signals were counted in 100 nuclei in each case with a 63x objective. AURKB and TP53 deletion was defined at copy numbers ≤0.8 per chromosome 17 based on earlier measurements on normal tissue sections, elevated copy number was stated higher than 2 gene copies per chromosome 17.

Chromosome 17 polysomy was stated at ≥2.3 CEP17 signals per nucleus.

AURKB gene amplification couldn't be observed in any Aurora B overexpressing samples.

On the contrary, loss of 17p13.1 was occasionally demonstrated due to the FISH signal loss relative to chromosome 17 signals. We observed TP53 signal deletion in 10 cases which was associated with the loss of the AURKB locus in 6 cases. AURKB and TP53 deletion was defined at copy numbers ≤0.8 per chromosome 17.

A strong correlation was observed between the mean copy numbers of AURKB and TP53 genes (r=0.73).

No direct association between AURKB/TP53 deletion and HER2 gene status could be stated. The loss of one of the AURKB and/or TP53 alleles was obvious in only 4/12 (33.3%) of the HER2 amplified cases.

Chromosome 17 copy number changes were seen in 19/50 cases (38 %). The range was between 2.3 and 3.9 copies/cell nucleus.

The copy number of chromosome 17 was found in AURKB and TP53 codeleted cases between 2.44-3.9 (mean: 3.18, SD: 0.18) all of the samples showed aneusomy; and between 1.6-3.19 in cases with normal gene copy numbers. The difference was statistically significant ($p<0.0001$).

Correlation between Aurora B expression and chromosome 17 copy number

To establish the possible connection between Aurora B expression and chromosome 17 copy number alterations we have studied the copy number changes in relation with CEP17 and 17p13.1 status.

Neither the Ki67, nor the Aurora B expression showed a direct correlation with chromosome 17 copy number changes.

A weak positive correlation was found between AURKB copy number and Aurora B expressing cell fractions (R: 0.26).

In cases with normal AURKB gene copy number the Aurora-B expressing cell fraction was in the range of 0-35 (mean=6.47±9.26) and 0-10 (mean=3.83 SD:3.44) in cases with gene copy number losses, however, the difference was statistically not significant (p=0.5). Further, AURKB/TP53 codeleted breast carcinomas showed an AMI between 0-0.25 (mean: 0.15 SD: 0.1), while cases with intact 17p were between 0-1 (mean: 0.36, SD: 0.3) for the same parameter (p= 0.5, not significant).

DNA content could not be measured on invasive breast carcinoma samples, nuclei isolation was not successful.

Statistically significant changes in Aurora B kinase expression in relation to 17p13.1 copy number could not be shown (p= 0.1).

Further, AURKB/TP53 codeleted breast carcinomas showed an AMI between 0-0.25 (mean: 0.15 SD: 0.1), while cases with intact 17p were between 0-1 (mean: 0.36, SD: 0.3) for the same parameter (p= 0.1, not significant).

Cases displaying very high AMI values (≥0.8) presented slightly higher signal AURKB FISH copy numbers (mean 1.1 SD: 0.2) in contrast to tumors with AMI ≤0.3 the majority of which had copy numbers below 1 (mean 0.9 SD: 0.2) (p= 0.004).

Aurora kinase B expression on aggressive B-cell lymphoma samples

Fifty patients with aggressive B-cell lymphoma were recruited to the study.

On the basis of immunohistochemical features five subgroups were identified: 29 cases were classified as DLBCL, non-germinal center B-cell-like (non-GCB), 14 cases as DLBCL germinal center B-cell-like (GCB), 3

cases as ALK+ large B-cell lymphoma (B-ALCL), 3 cases as primary mediastinal large B-cell lymphoma (PMBL) and 1 case as B-cell lymphoma, intermediate between DLBCL and classical Hodgkin lymphoma.

The Aurora B expressing cell fraction was observed between 23-36.5% (mean 30.45±5.06 SD) in control germinal centers of follicular hyperplasia samples and the same value was found between 5-70 % (mean 28.2±15.32 SD) in aggressive B-cell lymphoma samples.

The non-neoplastic control group presented significantly higher proliferation rates compared to B-cell lymphoma samples; MIB-1 positivity was seen between 20-95% (63.6±15.9, p value: 0.008) and the H3S10P mitosis index was found between 28-418/10 HPF (112.62±79.2, p value: 7.84×10^{-8}) the same values in control cases were 69-87% (mean 77.8±4.99 SD) and 183-459/10 HPF (mean 286.4±85.32 SD).

As shown in invasive breast carcinoma samples, Aurora B was found to be strongly influenced by intensive cell proliferation, highlighted by the MIB-1 immunostaining (regression coefficient R=0.7 in lymphoma and 0.5 in follicular hyperplasia samples).

As we assumed a basic effect of the cycling compartment on Aurora positivity the relative Aurora B expression was determined by the introduction of the AMI index for each sample. This value varied between 0.31-0.48 (mean 0.39±0.06 SD) in the control and 0.17-0.85 (mean 0.44±0.2 SD) in aggressive lymphoma cases p=0.44, statistically not significant).

The cut off for relative Aurora B overexpression was stated when the AMI value was higher than 0.5 on the basis of data calculated in the control group (mean AMI+2×SD). According to this formula Aurora B overexpression could be stated in 13/50 B-cell lymphoma cases (26%).

These cases showed statistically significant raise in mitotic activity (p value=0.016) compared to the lymphoma samples with AMI values $\leq$0.5. A subset (N=8 cases) of Aurora B overexpressing cases showed extremely high AMI value (AMI$\geq$0.75). In these latter cases the mitotic activity was measured even higher than the rest of the samples with Aurora B overexpression despite of similar MIB-1 positive fractions.

Regarding AMI no significant differences were shown between aggressive B-cell lymphoma subgroups either. The highest mean AMI value was found in B-ALCL; however, only 3 cases were classified into this group.

Survivin – upstream regulator of Aurora B

Survivin expressing cell fraction was found in the range of 28.5-61% (45.5±11.22) in the control and 10-85% (39.54±20.69) in lymphoma samples, no significant differences could be shown. However, a strong correlation was found between the amount of survivin and Aurora B expressing cell fractions in both the control and the B-cell lymphoma groups (R-values 0.73 and 0.7, respectively). Aurora B overexpressing lymphomas showed a significantly higher survivin positive fraction (p=0.0012).

Mitotic activity defined by phospho-histone H3 as a downstream indicator of Aurora B function

Exact identification of mitoses in histological slides was done by phospho-histone H3 immunostaining, which selectively highlights mitotic figures as strongly labeled objects. Compared to the reactive germinal centers lymphoma samples presented with a significant decrease in phosphorylated histone H3 expression (p-value= 7.84×10^{-8}).

However, a weak positive correlation was observed between Aurora B expressing fractions and phosphorylated histone H3 positive mitotic forms in the control samples with a correlation coefficient (R) of 0.26. This coefficient was found 0.41 in B-cell lymphoma.

Moreover, a significant difference was found between lymphoma groups with different AMI. Overexpression of Aurora B was associated with higher mitotic rates when AMI cut off was set >0.5 and >0.75 (p values 0.06 and 0.02, respectively).

Copy number alterations of AURKB, TP53 and chromosome 17

Copy numbers of the AURKB and TP53 in the locus 17p13.1 as well as chromosome 17 were evaluated by FISH in histological slides for each sample.

No direct association between Aurora B expressing cell fraction and AURKB gene copy number could be seen. The AMI was found between 0.16-0.86 (0.52±0.25) in cases with 17p13.1 loss, 0.19-0.83 (0.43±0.2) in aggressive B-cell lymphoma samples with intact 17p13.1 loci and 0.31-0.48 (0.39±0.06) in control reactive lymph nodes, statistically significant differences were not found (p values 0.39 and 0.15, respectively). On the other hand, we observed the loss of AURKB locus in 4/50 lymphoma cases (8%) with the simultaneous loss of TP53. Additional 7 cases (14%) had shown the loss of TP53 gene alone.

In lymphoma cases with TP53 and AURKB allelic loss the Aurora B expressing cell fraction was in the range of 10-60% (33.75±18.5), the same range was 5-70% (27.18±14.75) in cases with intact 17p13.1 regions and 23-36.5 % (31.27±4.65) in control cases. Differences were statistically not significant (p values 0.42 and 0.64, respectively).

TP53 loss was detected in all B-ALCL cases (3/3, 100%), in 3/29 (10%) cases with DLBCL non-GCB and in 2/14 (14%) samples with DLBCL GCB features.

Simultaneous loss of AURKB and TP53 was seen in 2/29 (6.9%) samples of non-GCB and 2/14 (14%) samples of GCB subclasses. The 17p13.1 region proved to be intact in all PMBL samples (0/3).

Ploidy status was addressed by the analysis of chromosome 17 copy numbers determined by the same FISH approach. The overall rate of aneusomy chromosome 17 was 46/50 (92.0%); disomy was seen in only 4 lymphoma cases (2 DLBCL GCB and 2 DLBCL non-GCB). Hyperdisomy was seen in 12 cases, trisomy in 25 cases and in additional 9 cases tetrasomy was observed. The highest mean copy numbers of chromosome 17 per nucleus were seen in cases with 17p13.1 loss.

On the basis of mean AMI value of follicular hyperplasia samples (0.39±0.056) 3 lymphoma groups were determined: lymphomas showing low AMI values (AMI≤0.28); normal AMI values (0.28<AMI>0.5) and Aurora B overexpressing samples with high AMI values (AMI ≥0.5). A subset of Aurora B overexpressing cases showed extremely high values (AMI≥0.75). In Aurora B overexpressing aggressive B-cell lymphoma samples the mean chromosome 17 copy number per nucleus was found between 1.9-3.56

(2.73±0.5). Statistically significant differences between aggressive B-cell lymphoma groups were not detected.

Correlation between Aurora B expression and patient survival

We evaluated the potential effect of Aurora B overexpression on patient survival in our sample group of 50 aggressive B-cell lymphoma patients. The median follow-up time was 34.44 months (range: 0.97-127.1).

Statistically significant difference in overall survival time regarding Aurora B relative overexpression could not be shown.

Regarding chromosome 17 copy numbers patient samples showing chromosome 17 hyperdisomy associated with shorter survival rate, the difference was not statistically significant (p=0.27).

DISCUSSION

Mitotic kinases, such as the Aurora kinase B are potential biomarkers for chromosome instability and mitotic errors leading to aneusomies and aggressive tumor phenotype.

The expression of Aurora B can be studied in tissue conditions by immunohistochemistry, as described previously.

The activity of Aurora-B in normally growing cell populations is limited to the G2/M phase of the cell cycle. When evaluated independently as a single variable, the proportion of the Aurora B expressing cells was reported to be increased in malignant tumors, also described as overexpression. However, specific reasons including genetic aberrations or gene expression deregulation were not described so far.

Aurora B expression in evaluated samples

According to our results Aurora B is overexpressed in a significant portion of aggressive B-cell lymphoma and invasive breast cancer samples.

We observed, that Aurora B expression is strongly correlates with cell proliferation activity. The correlation coefficient (R) was 0.77 in breast cancer, 0.5 in B-cell lymphoma and 0.77 in follicular hyperplasia samples.

Theoretically, together with the total proliferative activity, the G2 and M phase fractions are also expected to be proportionally increased. We assume that the elevated fraction of Aurora B immunopositive cells is mainly determined by the proportional increase of the G2/M phase where the kinase is expressed in a function specific manner. Therefore, the relative expression of the Aurora B protein is better reflected by the Aurora B/MIB-1 index which represents the Aurora B expression in relation to the whole proliferative fraction of the tumor.

To consider cell kinetic aspects the relative kinase expression within the proliferative fraction (AMI index) was calculated for both the non-neoplastic and aggressive clonal B-cell proliferations. Overexpression in lymphoma was stated when the AMI exceeded 0.5 (value exceeding the mean +2×SD of the control non-neoplastic samples). The cut off value indicated, that over the half of the cycling cell fraction was positive for Aurora B which was definitely

higher than regularly occurring G2/M phase fractions and could never be observed in the control samples. This phenomenon is rarely seen on breast carcinoma samples. When evaluated independently as a single variable, the proportion of the Aurora-B expressing cells was reported to be increased in malignant tumors, often interpreted as overexpression. However, biological reasons, such as specific genetic aberrations or gene expression deregulation were not described so far. According to our results the expression of Aurora kinase B is frequently increased in breast carcinoma and lymphoma but strongly dependent on cell proliferation. We assume that the elevated fraction of Aurora-B immunopositive cells is mainly determined by the proportional increase of the G2/M phase where the kinase is expressed in a function specific manner.

Aggressive tumors excluded by Her2 positivity/steroid receptor negativity showed a significantly higher Aurora-B protein expression but following the relativation to the proliferating cell fraction (AMI) they were not significantly different. As a result of detailed evaluation no clear impact of the Aurora-B overexpression, determined by IHC could be stated following the correlation with the most common variables in breast carcinoma.

Cytogenetic background of Aurora B overexpression

We were also looking at potential genetic causes of the overexpression, however, FISH analysis of the AURKB locus did not present a gain or amplification at this region.

On the other hand, losses in one of the AURKB loci could be demonstrated in few cases: in 4 aggressive lymphoma and 6 invasive breast carcinoma samples AURKB loss could be demonstrated. The copy loss was not associated with obvious changes in kinase expression. Losses demonstrated by FISH also involved the TP53 locus situated in close proximity (517 kbp) to the AURKB. However, the TP53 copy number was not strictly associated with AURKB as 7 more cases appeared with TP53 loss alone in aggressive B-cell lymphoma and 4 of breast carcinoma samples.

Our cytogenetic studies also represented a strong correlation between AURKB and TP53 copy alterations (R: 0.73).

Chromosome 17 copy number alterations

Chromosome 17 aneuploidy was frequently observed in breast carcinoma and lymphoma samples as well. Previous reports showed a stron correlation between tumour aggressiveness and aneusomy. As described previously chromosome 17 aneusomy by FISH is correlated with aneuploidy studying by flow cytometry.

According to our data chromosome 17 aneusomy is frequently observed in aggressive B-cell lymphoma: 46 of 50 cases showed copy number alterations. The highest chromosome 17 copy numbers were seen in B-ALCL, all cases were associated with TP53 deletion.

According to our findings on breast carcinoma samples, losses at 17p13.1 and chromosome 17 polysomy determined by FISH analysis correlated significantly. Although the number of the TP53/AURKB codeleted cases was low these findings gave rise further ideas regarding the generation and survival of malignant cell clones with 17p13.1 locus deficiency. A synergic effect of the two genes involved by the codeletion at the mentioned region can be speculated: loss of AURKB may serve for regulatory deficiencies in the chromosome passenger complex leading to mitotic errors while p53 deficiency helps the cell to survive due to insufficient activation of the intrinsic apoptotic pathways. These parallel effects finally increase the complexity of mitotic abnormalities and generate aneuploid cell populations.

Aurora B as a mitotic regulator

Aurora A and B were shown to physically interact with histone H3 indicating their responsibility in phosphorylation at Ser10 and 28 initiating the mitotic condensation during G2/M transition. Experimental inhibition of Aurora B kinase function resulted in a dramatic reduction of Ser10 phosphorylation. On the opposite, the kinase overexpression caused increased phosphorylation at Ser10 associated with incomplete chromosome condensation and misalignment in the form of abnormal mitoses in HeLa cells.

The decrease of mitotic cell numbers identified by mitotic histone H3 phosphorylation was obvious in lymphoma compared to reactive germinal centers despite of the relative increase in Aurora B expression, a result clearly

underlining the significance of the total cycling compartment when comparing populations for phase specific features. These and earlier data also indicate to a highly intense proliferative activity due to immunological stimuli in the reactive germinal center that is only rarely reached in aggressive lymphoma.

On the other hand, mitotic activity correlated with Aurora B expression and increase in phospho-H3 labeling was obvious in Aurora B overexpressing lymphoma samples compared to cases with normal kinase expression. One possible explanation for this can be the induction of early chromatin condensation induced by the progressive phosphorylation of H3 in the terminal G2/M phase due to kinase overexpression and activation. A key element of Aurora B overexpression could therefore be the timely reorganized G2/M transition leading to acceleration of the premitotic steps. As a consequence, repair functions of the G2/M checkpoint remain potentially incomplete contributing to the persistence of errors at both DNA and cell division.

We also performed studies to evaluate up- and downstream interactions of Aurora B. Survivin is a G2-phase protein regulating the proper function of the CPC and activating Aurora B [6]. Our IHC based results suggest that survivin precedes the expression of Aurora B in the cell cycle and is in good correlation with their sequential occurrence. However, clear signs of survivin deregulation affecting Aurora B overexpression or dysregulation could not be stated at this level.

Clinicopathological findings

50 patients with invasive breast carcinoma were recruited to the study. 45 tumour samples were showing invasive ductal carcinoma morphology and the remaining five cases were invasive lobular carcinomas. ER and PR positivity was observed in 36 and in 27 cases, respectively. 16 cases were c-erb-B2 expression negative. We have observed HER2 gene amplification in 16 cases.

Fifty patients with aggressive B-cell lymphoma were recruited to the study.

On the basis of immunohistochemical features five subgroups were identified: 29 cases were classified as DLBCL, non-germinal center B-cell-like (non-GCB), 14 cases as DLBCL germinal center B-cell-like (GCB), 3 cases as ALK+ large B-cell lymphoma (B-ALCL), 3 cases as primary

mediastinal large B-cell lymphoma (PMBL) and 1 case as B-cell lymphoma, intermediate between DLBCL and classical Hodgkin lymphoma.

Statistically significant differences between biological subgroups could not be stated.

Statistically significant difference in overall survival time regarding Aurora B relative overexpression could not be shown. The highest AMI values were seen in B-ALCL, it was associated elevated mitotic activity and chromosome 17 copy numbers.

Direct correlation between genetic instability and Aurora B expression alone, as an independent biomarker could not be stated. According to our data, H3S10P expression, as an indicator of Aurora B kinase function may be more applicable for prediction indirectly genetic intability.

SUMMARY

The main goal of this work was to study the effect of Aurora kinase overexpression on cell ploidy and tumourigenesis. 50 invasive breast cancer, 50 diffuse large B-cell lymphoma and 10 reactive lymph node sample were recruited in the study.

Our main findings:

1. The expression of Aurora B and its binding partners can be studied between histological conditions.
2. Immunohistochemistry and FISH are reproducible methods on tissue microarrays.
3. Because of the significant correlation with the overall cell proliferation rate, the overexpression of Aurora B could not be stated on the basis of kinase expressing tumor cell fractions alone. The relative expression of the Aurora B kinase is better reflected by the AMI index which represents the Aurora B expression in relation to the whole proliferative fraction of the tumor. The higher relative Aurora B expression associated with higher mitotic activity in B-cell lymphoma.
4. FISH analysis of the AURKB locus did not show any gains or amplifications in the samples analyzed. On the other hand, we have observed the loss of the gene is breast carcinoma and lymphoma samples as well. A strong correlation was shown between AURKB and TP53 copy numbers: AURKB loss was associated with TP53 deletion in all samples. According to our results on breast carcinoma, losses at 17p13.1 and chromosome 17 aneusomy determined by FISH showed a statistically significant correlation.
5. Our study presents the frequent occurrence of aneusomy chromosome 17 in breast carcinoma and B-cell lymphoma samples. Chromosome 17 aneusomy evaluated by FISH is correlated with aneuploidy studying by flow cytometry.
6. Direct correlation between kinase expression and ploidy could not be shown.
7. The highest Ami values was seen in B-ALCL samples, it was associated with high chromosome 17 copy numbers and mitotic activity.

The damaged Aurora B kinase function serve for regulatory deficiencies in the CPC complex leading to mitotic errors, while p53 deficiency helps malignant cells to survive due to insufficient activation of the intrinsic apoptotic pathways. The upregulation of Aurora kinase B function results an error in an important mitotic checkpoint, thus resulting in induction of aneuploid cell populations.

These parallel effects finally increase the complexity of mitotic abnormalities and generate aneuploid cell populations.

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List of publications related to the dissertation

1. **Hegyi, K.**, Lønborg, C., Mónus, A., Méhes, G.: One-Day FISH Approach for the High-Speed Determination of HER2 Gene Copy Status in Breast Carcinoma.
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OTHER PUBLICATION RELATED TO THIS PUBLICATION

Hegyi K, Bedekovics J, Dócs O, Irsai G, Gergely L, Beke L, Méhes G: **Mitotic kinase Aurora B is frequently overexpressed in aggressive B-cell lymphoma** (Pathology International, submitted)

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