SHORT THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

GENETIC AND EPIGENETIC BACKGROUND OF MELANOMA PROGRESSION

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The Examination takes place at the Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen 10th July 2014, 11 am

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The PhD Defense takes place at the Lecture Hall of Bldg. A, Department of Internal Medicine, Faculty of Medicine, University of Debrecen, 10th July 2014, 1 pm

INTRODUCTION

Malignant melanoma is one of the most aggressive skin cancers with a constantly increasing incidence. As estimated by the World Health Organization worldwide number of newly diagnosed skin cancer cases is between 2 and 3 million each year, of which 132,000 are melanoma. Additionally, in most western countries, the incidence of this malignancy doubles roughly every decade. Melanoma is a highly metastatic disease and as soon as the first distant metastasis appears, the melanoma becomes one of the most aggressive types of metastatic, chemoresistant lesions with poor survival rate.

The spectrum of somatic alterations in melanoma includes both genetic and epigenetic events that act in concert to promote tumour progression. The best described factor involved in epigenetic inheritance is DNA methylation, a covalent modification that mainly acts in cytosines.

It is important to distinguish between genome-wide hypomethylation and localised hypermethylation. DNA hypermethylation (known also as localized or regional hypermethylation) is usually strictly localised to the transcriptionally active gene regions and promoters and directly inhibits gene expression.

In addition to the rapid progress that has been made in studying promoter hypermethylation at the single-gene level, only two groups have attempted to conduct array-based experiments to identify the methylation pattern of thousands of gene promoters. Regrettably, one group has focused only on comparing the methylation level of primary invasive melanomas with benign melanocytes and has clearly identified a group of genes in a statistically powerful interpretation that can be used

to discriminate naevi from melanomas based on their methylation signature.

While most groups are studying extensively promoter-related hypermethylation, the importance of genome-wide demethylation or hypomethylation remains underestimated. However, these phenomena might also reflect important epigenetic alterations due to their ability to cause genetic instability. Genome-wide hypomethylation is characterised by the overall loss of 5-methyl-cytosines, which is believed to correspond to the loss of methylcytosines of repetitive transposable elements. During evolution, these elements integrated into the human genome and became protected from transcription due to their higher levels of methylcytosines. Considering the abundance of repetitive elements, it is clear why scientists generalise the loss of their 5-methyl-cytosine content to the ’whole genome’.

Integrative genomic approaches have recently afforded the opportunity to profile multiple types of alterations on a genome-wide scale in the context of gene expression and function. For obtaining a more generalized insights into the molecular mechanisms that might be responsible for the aggressive phenotype associated with the observed gene expression signature of ulcerated melanomas, we performed an extensive genome analysis by integrating DNA copy number and methylation results with the gene expression changes revealed in ulcerated vs. non-ulcerated melanomas. The main purpose of this part of the present study was to give functional relevance to the copy number events by providing a statistically powerful integrated interpretation of gene expression changes and copy number alterations.

OBJECTIVES

In an effort to further advance our understanding of the relationship between primary malignant melanoma progression, our aims were as follows:

Investigating of transposable DNA methylation:

- to assess global DNA methylation status of a set of primary melanomas with different biologic behavior by investigating the methylation level on the 6 CpG sites of transposable *LINE 1* sequences using Na-bisulphite pyrosequencing.

Studying of localized methylation patterns

- to draw DNA methylation-based distinction among diverse melanoma subtypes using a Bead Assay specific for more than 800 cancer related genes.
- to investigate the relationship between DNA methylation patterns and distinct types of somatic alterations, including the most frequent mutations and DNA copy number changes.

Performing integrative genome approaches:

- to characterise genomic hotspots with functional relevance and define cis- and trans acting copy number alterations on a genome-wide scale that act in the establishment of disturbed gene expression pattern in primary melanomas.
- to measure the correlation between gene expression and regional DNA methylation for genes that we had available DNA methylation profiling data.

MATERIALS AND METHODS

MELANOMA SAMPLES

Forty-six primary melanomas were involved in our studies. Tumour tissues were obtained from the Department of Dermatology, University of Debrecen, Hungary. All human studies were conducted in accordance with principles outlined in the Declaration of Helsinki and were approved by the Regional and Institutional Ethics Committee of the University of Debrecen Medical and Health Science Centre and was conducted according to regulations. Written informed consent was obtained from each patient.

High-quality total RNA was prepared from primary melanoma tissues using the RNeasy Mini kit according to the protocol of the supplier (Qiagen, GmbH, Germany). The obtained RNA concentrations were measured using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA). RNA sample integrity was determined with the Agilent 2100 Bioanalyser using the RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA, USA). All RNA samples exhibited a 28S/18S ribosomal RNA ratio greater than 1.5.

The G-spin™ Genomic DNA Extraction Kit (Intron, Korea) was used to isolate high molecular weight DNA samples from primary melanomas according to the protocol provided by the manufacturer. To determine the quantity of DNA obtained, we used a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware USA). DNA integrity was verified via 1.2% agarose gel electrophoresis.

LABORATORY TECHNICS

Na-bisulphite pyrosequencing

Genome hypomethylation was investigated primary melanomas (n=46) by pyrosequencing 6 CpG sites at the *LINE 1* (long interspersed nucleotide element 1) transposable sequence being integrated in the human genome. Primers were specific for the promoter region of the 5-6.000 bp long transposable sequence and Q-CpG Software (Biotage, Sweden) was used for quantitative assessments. Prior to the pyrosequencing by PQS System (Biotage, Sweden), DNS samples were treated with Na-bisulphite (EZ DNA Methylation-Gold kit, Zymo Research, CA, USA), which converts non-methylated cytosines into uracil but does not affect the methylated nucleotides; therefore results can be referred ad C/T polymorphisms.

DNS methylation Bead Assay

The quantitative methylation status of the 1,505 CpG sites corresponding to 807 cancer-related genes (mainly at the promoter regions) was determined using the Illumina GoldenGate Methylation Assay (Illumina, San Diego, CA, USA) on bisulphite-treated DNA samples corresponding to 42 primary melanomas. This technology utilizes 3 μm silica beads which are replicated ~30 times on the array.

M-values, the logistically transformed Avg-Beta methylation values were used for statistical approaches. In agreement with the literature, probes corresponding to the sex chromosomes were excluded to avoid any sex-specific bias. The probes with detection P values exceeding 0.01 in more than 10% of the specimens were removed from the analyses to exclude non-biological differences.

Quantitative RT-PCR

Based on our DNA methylation results, the gene expressions of *FGFR3, MCAM* and *IL8* were measured using quantitative real-time PCR (QRT-PCR) with the ABI Prism® 7900HT Sequence Detection System (Applied Biosystems, Carlsbad, California, USA). Reverse transcription (RT) was carried out on total RNA (600 ng) using the High Capacity cDNA Archive Kit, according to the protocol of the supplier (Life Technoligies Corporation, Carlsbad, California, USA). Predesigned TaqMan® Gene Expression Assays (Life Technologies Corporation, Carlsbad, CA, USA) were used to perform QRP-PCR for the abovementioned three genes. QRT-PCR data were analysed using the Livak method with GAPDH as the reference gene and nevi collected from three different individuals and melanocyte as calibrator samples.

Fluorescence in situ hybridization (FISH)

FISH experiments were performed on touch preparations of primary melanoma samples. Multicolour FISH probe were specific for *RREB1* (Spectrum Red, red fluorescence) *MYB* (Spectrum Gold, yellow fluorescence) and *CCND1* genes (Spectrum Green, green fluorescence) and centromere region of chromosome 6 (Spectrum Aqua, blue fluorescence). Slides were evaluated with a Zeiss Axio Imager Confocal Microscope.

Array Comparative Genome Hybridization and integrated genome analysis

Tiling Array CGH HG18 CGH 4x72K WG Tiling v2.0 experiments (Roche NimbleGen core facility, Reykjavik, Iceland) were done in DNA samples isolated from primary melanomas (n=26).

Array CGH results were analysed by Nexus 5.1 Copy Number Software (BioDiscovery, CA, USA). The GISTIC algorithm was used to identify regions containing a statistically high frequency of copy number aberrations compared to the "background" aberration frequency. This function is most appropriate for cancer samples, as it was designed using a cancer dataset. Array CGH results were used for integrated genome analysis (see Statistical methods section).

STATISTICAL ANALYSES

Analysis of genome wide methylation patterns

Normality of *LINE 1* pyrosequencing results was checked by D'Agostino and Pearson omnibus normality test. To estimate the effect of the *LINE 1* methylation status on patient survival, the *LINE 1* methylation level represented by continuous variables was dichotomised at its cut-off value determined for each CpG site by the Receiver Operating Characteristic (ROC) curve analysis.

The clinical parameters including patient age and gender, Breslow thickness, histological subtype, metastasis formation during the 5-year follow up period and tumour surface ulceration were examined by the Cox proportional hazards regression model, and the effect of hypomethylation on the survival was presented as hazard ratios (HR) with corresponding 95% confidence intervals (CI). To control for the above-noted additional confounders, a stepwise regression was applied to select variables. The nonparametric Mann Whitney and Kruskal-Wallis tests with Dunn's Multiple Comparison Post-test were used to establish a connection between the *LINE 1* methylation status and clinical parameters.

For statistical analysis, GraphPad Prism 5.0 (CA, USA) and MedCalc (Ostend, Belgium) softwares were applied.

Analysis of localized methylation pattern

The DNA methylation results revealed by Illumina GoldenGate Methylation Assays were analysed by BRB Array Tools Software. To determine CpG sites which are differentially methylated between the melanoma subgroups, t-test or f-test were applied after multiple corrections. Results were verified by Principal Component Analysis. To determine the overlapping genes among the clinical subclasses, Venn diagrams were used.

To evaluate the KEGG-based gene networks disturbed by DNA methylation, we applied the Efron-Tibshirani Gene Set Analysis that uses 'maxmean' statistics to identify gene sets expressed differentially among predefined class.

The Cox proportional univariate approach was performed on each gene to test whether the methylation status of a particular gene significantly influences the survival at the $p < 0.05$ level.

Integrated genome analysis

To study the relationship between DNA copy number gains/losses and mRNA levels, we exported the median of the replicate probe $\log_2$ ratios and the expression values corresponding to the same genomic region for determining Pearson’s correlation. The genelist generated through this analysis was imported to the Nexus 5.1 package. We assessed the genomic locations that exhibited significant copy number losses and concentrations of downregulated genes simultaneously. In order to verify cis-acting and identify trans-acting CNVs, we performed Lasso-regression („LOL” - Lots of Lassos in “R” program language developed by Yuan et al.).

RESULTS

TRANSPOSABLE DNA METHYLATION PATTERNS OF PRIMARY MELANOMAS

We quantitatively assessed the global DNA methylation patterns of 46 primary malignant melanomas with differing clinical characteristics by measuring the methyl-cytosine content of the *LINE 1* transposonal sequence. Six distinct CpG sites on the *LINE 1* promoter region were measured by pyrosequencing. Kaplan-Meier curve analysis revealed significant differences between the hypomethylated and non-hypomethylated patients, with a decreased overall survival (OS) rate for the hypomethylated group for each CpG site. Notably, the hazard ratios (HR) were higher than 3.0 in each hypomethylated group for all six CpG sites.

The Cox proportional hazard regression model allowed for the control of additional clinical covariates as well as for the patient gender and age. As the stepwise regression method indicated metastatic potential (when primary melanomas develop metastasis during the five-year follow up period) that influences patient survival, this potential was included in the model. The adjusted HR values did not remain higher than 1.46 nor did the adjusted p-values remain significant.

As the stepwise method of Cox proportional hazard regression model suggested the direct relationship of the overall loss of 5-methylcytosine with metastatic potential, we built a logistic regression model with a stepwise selection for *LINE 1* hypomethylation and for the clinical parameters. For each CpG site, we found high odds ratios (OR) for the hypomethylated sample groups; however, the OR remained significant for CpG1. The Mann-Whitney test confirmed that the global

hypomethylation of all six CpGs was significantly associated with metastasis formation. Furthermore, significantly different levels of global methylation were detected among tumours without metastasis and melanomas with more than one metastasis over 5years.

REGIONAL DNA METHYLATION PATTERNS OF PRIMARY MELANOMAS

We studied the methylation patterns of Na-bisulphite converted genomic DNA samples isolated from 42 primary melanomas. Our main goal was to investigate the relationship between the distinct biological types of melanomas and the promoter methylation levels. Altogether, 111 differentially methylated genes were identified in the context of clinical predictors; however, during melanoma progression, hypomethylation was more characteristic compared to hypermethylation. Interestingly, each clinical subclass was characterized by individual DNA methylation pattern.

Due to its therapeutic importance, $BRAF^{V600E}$ mutation and its relationship with DNA methylation were also investigated. Apart from the clinicopathological characteristics of melanomas, BRAF^{V600E} mutation was found to be individually associated with the CpG methylation changes of 23 genes that are related to Cell Communication and ECM-receptor interaction networks.

Inverse relationships were found between DNA methylation and mRNA expression regarding *FGFR3, MCAM* and *IL8*, supporting the notion that the methylation pattern are functionally relevant to gene expression.

The effects of DNA methylation on patient survival were also investigated. After correction for the clinical characteristics, *KIT* gene was found to be significantly associated with poor patients' survival.

Our group performed a Tiling Array CGH, and, apart from highlighting common CN losses and amplification in the subgroups of primary melanomas, we demonstrated that 6q12-6q25.1 comprises a remarkable CN loss, harbouring two hypermethylated genes on 6q23, *EYA4* and *MYB1*. This result was measured and verified quantitatively by FISH experiments and provides evidence for Knudson's two-hit hypothesis at the level of CN loss and DNA hypermethylation.

Our Tiling Array CGH experiments showed another important feature: the CN alterations of chromosome 19 were only detected in advanced staged primary melanomas. Notably, the altered genomic regions encompass 19p13.2, which harbours the *DNMT1* gene (DNA Methyltransferase-1), which plays a role in the establishment and regulation of tissue-specific patterns of methylated cytosine residues.

INTEGRATED GENE EXPRESSION, GENE COPY NUMBER AND DNA METHYLATION ANALYSIS

The main purpose of this part of the present study was to give functional relevance to the copy number events by providing a statistically powerful integrated interpretation of gene expression changes and copy number alterations. Based on our aCGH analyses, we found more regions on chromosome 6q and only one region on chromosome 10q that showed significantly different loss of copy numbers between the two clinical subgroups. It is important to note that these regions were enriched for the downregulated transcripts and significantly

correlated with our previously defined gene expression results. There are a total of 36 genes in these two regions, among which we identified 10 downregulated transcripts using the Affymetrix microarray. It is important to note that these genes include *TPBG, PERP* and *UTRN,* which are involved in cell-cell and cell-matrix adhesion. *PERP* also functions as a p53-induced apoptosis effector molecule. Furthermore, *TPD52L1* participates in apoptosis followed by nuclear fragmentation. According to the literature, *IL20RA* is a tissue-specific Interleukin Receptor that is highly expressed in normal skin

While systematic correlation analysis among the copy number events and the corresponding genes captures cis- effects, it is also important to measure trans- acting copy number alterations which occur at a given genomic loci and can affect the mRNA expressions of distant genes. Based on our analyses, 6q27, 7q11 and 9p21 deletions as well as 6p25.3-p25.2, 17q22, 17q24.3 amplifications were proven to be associated with the impaired expression of distant genomic regions.

Furthermore, we correlated the gene expression data with the observed epigenetic changes in the same tumour. Of the 987 downregulated genes which were described previously, we found 45 common genes. A strong or medium inverse correlation between gene expression and methylation levels was detected in the case of 11 genes: *CDH13, DST, EPHB3, EPHB6, ETS2, FGFR2, FGFR3, ITGA2, JAG1, PTGS1, TIAM1*.

DISCUSSION

Despite the strong progress has been made in the field of melanoma epigenetics, the importance of genome-wide demethylation or hypomethylation remains underestimated. However, this phenomenon might also reflect important epigenetic alterations due to its ability to cause genetic instability. Furthermore, no methylation-based distinction has been drawn among the diverse primary melanoma subtypes.

To assess global methylation we measured the methylation level on the 6 CpG sites of *LINE 1* sequences in 46 primary melanomas in association with patients' survivals and the clinicopathological characteristics of specimens.

We demonstrate that *LINE 1* hypomethylation is accompanied by the shortened relapse-free survival of melanoma patients; however, Cox regression analysis shows a direct relationship between the overall loss of 5-methylcytosine and metastatic potential of primary melanomas, which is confirmed by Kruskal-Wallis tests with Dunn's Multiple Comparison Post-test showing that not only the presence but the number of metastases during the 5-year follow-up period is associated with the transposonal demethylation.

In melanoma, the presence of promoter related hypermethylation has previously been reported, however, no methylation-based distinction has been drawn among the diverse melanoma subtypes. Here, we investigated DNA methylation changes associated with melanoma progression and links between methylation patterns and other types of somatic alterations, including the most frequent mutations and DNA copy number changes.

Our results revealed that the methylome, presenting in early stage samples and associated with the $BRAF^{V600E}$ mutation,

gradually decreased in the medium and late stages of the disease. An inverse relationship among the other predefined groups and promoter methylation was also revealed except for histologic subtype, whereas the more aggressive, nodular subtype melanomas exhibited hypermethylation as well. The Breslow thickness, which is a continuous variable, allowed for the most precise insight into how promoter methylation decreases from stage to stage. Integrating our methylation results with a high-throughput copy number alteration dataset, local correlations were detected in the *MYB* and *EYA4* genes. With regard to the effects of DNA hypermethylation on melanoma patients' survival, correcting for clinical cofounders, only the *KIT* gene was associated with a lower overall survival rate. Considering the oncogenic status of the *KIT* gene that is targeted by imatinib therapy, our results are hard to interpret. Our contradictory result can possibly be due to another epigenetic mechanism discovered in 2009 and known as 5-hydroxy-methyl-cytosin (5-hm-C), the sixth base of the DNA. Compared to 5-methyl-cytosin (5-m-C), 5-hm-C adversely affects and enhances the gene expression.

Although the precise mechanism of action and regulation of 5-hm-C is being investigated; the presence of a new base in melanomas has also been demonstrated. Since detection methods used 2-3 years ago cannot distinguish between the two different types of methylation, the *KIT* gene hydroxymethylation was possibly detected, which can cause gene upregulation. The novel type of DNA methylation indicates the partial limitation of our results as well as all the experiments done before 2012 in melanoma. In the future, the extent of the *KIT* gene 5-hm-C should be investigated along with a revised survival analysis.

Our previous study identified a set of genes with decreased gene expression to be associated with less favourable

clinical outcome. Integrating our high throughput array CGH results with the aforementioned gene expression panel, we identified genomic hotspots on chromosome 6q and 10q, where copy number loss was associated with decreased gene expression. Most of the genes were involved in cell-cell and cell-matrix adhesion or apoptosis.

Besides defining cis-acting copy number alterations, our integrated genome analysis revealed trans-acting genomic locations and, to the best of our knowledge, provided first evidence for such phenomenon in malignant melanoma.

MAIN STAEMENTS AND RESULTS

The main purpose of the doctoral thesis was to provide a comprehensive genetic and epigenetic study to define somatic DNA alterations that contribute to the aggressive biological behavior of human primary melanomas.

Investigating genome-wide (transposable) DNA methylation:

- We demonstrated the strong influence of *LINE 1* transposable demethylation in the metastatic formation of primary melanomas during the follow-up period.

Studying regional (localized) DNA methylation:

- The methylome, presenting in early stage samples and associated with the *BRAF*^{V600E} mutation, decreased in the more advanced stages of the disease.
- Local coordinated allele loss and DNA hypermethylation was shown at the region of 6q22-q23 that encodes the *MYB1* and *EYA4* genes.
- We revealed that the 19p13.2 genomic region harboring *DNMT1* gene (DNA methylatransferase-1 responsible for the maintenance of methylation patterns during DNA replication) often suffers from DNA copy number loss in melanomas thicker than 4 mm.
- The DNA methylation changes of the *KIT* gene was significantly associated with shortened relapse-free survival in melanoma patients.

Integrative genomic analysis:

- A set of genes with copy number loss were defined in ulcerated malignant melanomas, which were significantly enriched on chromosome 6q and 10q. Most of the genes were involved in cell-cell and cell-matrix adhesion or apoptosis.

- The first evidence for trans-acting copy number changes was given in melanomas.
- The expression and methylation patterns of additional genes exhibited an inverse correlation, suggesting that transcriptional silencing of these genes is driven by epigenetic events.

In conclusion, we demonstrated the strong influence of genome-wide as well as regional DNA methylation changes on melanoma progression. Methylation pattern was demonstrated to be a part of an integrated apparatus of somatic DNA alterations. We identified functionally relevant molecular hotspots characterised by copy number losses and promoter hypermethylation that might indicate a poor clinical outcome of melanoma.

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List of publications related to the dissertation

1. **Ecsedi, S.I.**, Hernandez-Vargas, H., Lima, S.C., Vízkeleti, L., Tóth, R., Lázár, V., Koroknai, V., Kiss, T., Emri, G., Herceg, Z., Ádány, R., Balázs, M.: DNA methylation characteristics of primary melanomas with distinct biological behaviour.
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5. **Ecsedi, S.**, Tóth, L., Balázs, M.: Array CGH analysis of the rare laryngeal basaloid squamous cell carcinoma: A case report.
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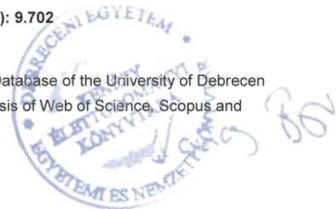

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International presentations related to the dissertation

Ecsedi S, Hernandez-Vargas H, Lima SC, Vizkeleti L, Toth R, Lazar V, Herceg Z, Adany R, Balazs M. Global hypomethylation and promoter related demethylation are associated with copy number loss of *DNMT1* gene and unfavourable clinical outcome in primary melanomas. 38th FEBS Congress, 6-11 July, 2013, Saint Petersburg, Russia

Ecsedi S, Lazar V, Vizkeleti L, Emri G, Rakosy Z, Adany R, Balazs M. Copy number variation and promoter methylation contribute to transcriptomic profiles associated with malignant melanoma progression. The 4th EMBO Meeting, 22-25 Sept, 2012, Nice, France

Ecsedi S, Vizkeleti L, Lima SC, Hernandez-Vargas H, Rakosy Z, Herceg Z, Adany R, Balazs M. DNA methylation pattern of malignant melanoma at wide panel of cancer related genes. The 2nd EMBO Meeting, 4-7 Sept, 2010, Barcelona, Spain

National presentations related to the dissertation

Ecsedi S, Hernandez-Vargas H, Herceg Z, Adany R, Balazs M. A daganat genom alterációi: epigenetikai és strukturális változások. VII. Conference of the Hungarian Association of Public Health Schools (NKE), 4-6 Sept, 2013, Kaposvár, Hungary

Ecsedi S, Vizkeleti L, Hernandez-Vargas H, Lima SC, Toth R, Lazar V, Herceg Z, Adany R, Balazs M. Transposonal hypomethylation and local demethylation of primary melanomas are associated with copy number loss of *DNMT1* and unfavourable clinical outcome. Hungarian Molecular Life Sciences 2013 Conference. 5-7 Apr, 2013, Siófok, Hungary

Ecsedi S, Vizkeleti L, Lima SC, Hernandez-Vargas H, Herceg Z, Adany R, Balazs M. Hypo- és hypermetilácós epigenetikai mintázatok humán malignus melanomákban. VI. Conference of the Hungarian Association of Public Health Schools (NKE), 5-7 Sept, 2012, Budapest, Hungary

Ecsedi S, Lima SC, Hernandez-Vargas H, Lazar V, Vizkeleti L, Rakosy Zs, Herceg Z, Adany R, Balazs M. Kópiaszám variabilitás és epigenetika változások primer melanomákban. V. Conference of the Hungarian

Association of Public Health Schools (NKE), 31 Aug - 2 Sept, 2011, Szeged, Hungary

Other presentations

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