Analysis of the clinicopathological and the prognostic factors of melanoma and pregnancy associated melanoma

PhD thesis

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1. Introduction

Pregnancy-associated melanoma (PAM) is one of the most often diagnosed cancers during gravidity, representing a distinct clinicopathological entity among melanomas with unique therapeutic challenges. One third of women diagnosed with melanoma are in their reproductive age. The exact incidence of PAM is not known, but the number of the diagnosed melanomas is 2-5/1000 000 gravidity.

The higher incidence of melanoma encountered in women, but the more unfavorable prognosis found in men, moreover the presence of classical estrogen receptors (ER) in the tumor, all suggest the hormone sensitivity of melanoma.

Estrogens and their most potent form, 17β-estradiol (E2) have various roles in physiological processes, such as reproduction, cell growth, development and differentiation, but also influence tumorigenesis in some hormone dependent tissues as well. Estrogens exert their genomic (classical) effects through ERα and ERβ, soluble receptors shuttling between the cytoplasm and the nucleus, and imbalance between ERα and ERβ expression may lead to tumor progression in some estrogen sensitive tumors.

Besides the classical estrogen signalling, E2 can also activate the alternative mitogen activated protein kinase (MAPK), extracellular signal-regulated kinase (Erk)-1/2 pathway through G protein-coupled oestrogen receptor (GPER). This third type of ER was detected as an

estrogen binding receptor at the beginning of 2000's, and it allows cells lacking classical ERs to respond to estrogen both in vivo and in vitro.

2. Objectives

1) As melanoma is one of the most common diagnosed malignancy during gravidity, we decided to examine its frequency and tumor stage distribution of PAM patients at the Department of Dermatology, Venereology and Dermatooncology of the Semmelweis University.

2) The literature is inconsistent about the clinicopathological features and the survival rate of PAM and non-pregnancy associated melanoma (NPAM). We aimed to analyse the clinicopathological factors as used in the routine diagnostics, as well as the survival rate of PAM and the age- and stage matched NPAM patients.

3) The presence of classical estrogen receptor, ERβ has described in melanoma. Our aim was to investigate the frequency of ERβ in PAM and NPAM, its presence in melanomas diagnosed during gravidity or postpartum, as well as in NPAM between genders.

4) GPER has been intensively examined in tumor pathology, and it is considered now as a prognostic factor in some hormone sensitive

cancers. As it has not been studied in human melanoma tissue yet, we aimed to investigate its expression and subcellular localization in human melanoma. Our purpose was to determine, the presence and the distribution of the receptor in PAM and NPAM, as well as in PAM diagnosed during gravidity or postpartum, and in NPAM in males and females.

5) In primary melanoma, the Breslow thickness, the mitotic rate and the ulceration are crucial clinicopathological factors determining the prognosis. We also examined, how are these factors associated with the presence of estrogen receptor subtypes in melanoma.

6) We aimed to investigate the association between the following features and the ER subtypes: *de novo* and naevus associated melanomas, the anatomical localization of the tumor, the histological subtype, the presence and the pattern of the peritumoral lymphocyte infiltration (PLI).

7) Our aim was further to determine, whether ER expression has a prognostic value in melanoma, and how is the soliter or the simultaneous expression of ERβ and GPER influence the disease free survival.

3. Methods

Selection of melanoma patients by retrospective analysis of the clinicopathological characteristics

In the first part of our research, we checked the data of the Oncodermatological Department of the Dermatology Clinic between 1st of January 2003 and 31th December 2014, and identified all together n=34 PAM (n=17, during gravidity and n=17 postpartum, age: 32.5±5.6 year). We further selected 64 non-pregnancy associated melanoma (NPAM) patients as controls (women (NPAM-W)(n=32) and men (NPAM-M)(n=32) samples in the same age and clinical stage.

Clinicopathological data collection

During the comparison of the clinicopathological data, we analysed the primary tumor characteristics used in the routine diagnostic clinicopathology (histological subtype, Breslow thickness, Clark level, tumor cell type, mitotic rate, peritumoral lymphocyte infiltration (PLI), vascular invasion, ulceration, necrosis, regression, the presence/absence of satellites, serum S-100 protein level, BRAF mutation analysis).

Statistical analysis

Statistic tests were carried out in IBM SPSS Statistics for Windows, Version 22.0 Armonk, NY: IBM Corp. For continuous variables, PAM and NPAM groups were compared by independent sample t-tests (age at diagnosis) and Mann-Whitney U test (Breslow thickness and mitotic rate). Between-group differences in discrete variables (e.g. Clark level, PLI, tumor cell types, histologic subtype) Chi-square test or Fisher's exact test were used. A Kaplan-Meier survival analysis was carried out using Log-rank test. All the statistic tests were two-sided and $p<0.05$ was considered statistically significant.

The immunhistochemical analysis of classical and non-classical estrogen receptors and the association between their expression and the clinicopathological factors

For the analysis of the ERs, 4 more patiens were added to the available study population, as the patients diagnosed with pregnancy-associated melanoma till 31th of December 2015 were also included (altogether n=38 PAM was examined, 8 patients in the first, 8 in the second and 4 in the third trimester and 18 in the postpartum group).

We further selected altogether 43 age- and Breslow thickness matched NPAM patients, including women (n=22) and men (n=21), with similar clinicopathological characteristics. The important

clinicopathological factors of PAM and NPAM groups (Breslow thickness, *de novo*- or naevus associated melanoma, the localization of melanoma, histological subtype, mitotic rate, ulceration, PLI, tumor infiltrating lymphocyte (TIL), mean follow-up time) in the three patients groups (NPAM-men, NPAM-women and PAM) before the analysis of the receptor expression were also compared.

Immunohistochemical and immunofluorescent analyses

Protein expression of ERα, ERβ and GPER was evaluated by immunohistochemistry (in a part of the cases immunofluorescent analysis was also performed) on archived, formalin fixed, paraffin embedded melanoma tissue samples of 81 patients. In cases of melanoma diagnosed during gravidity (n=20), the progesteron receptor (PGR) expression was also evaluated. A combined scoring was used for classifying the nuclear and cytoplasmic positivity. The intensity of the staining was scored as: (- (0), + (1), ++ (2) or +++ (3). According to the relative percentage of stained tumor- or inflammatory cells, (0% (0), <10% (1), 10-60% (2) and > 60% (3) points were given. The mean of scores by analysing 10 fields of view at 40x magnification was combined for the final score in each case (minimum 0, maximum 6 points). Only scores between 2 and 6 were considered as positive.
TIL and PLI were also classified by the abovementioned combined scoring system. When PLI was present, according to its localization five

pattern was considered according to the distribution of the lymphocytes: *leading edge* (lymphocytes are along the tumor, in the tumor growth zone), *shoulder effect* (lymphocytes are located on both side of the tumor mass), *perivascular* (lymphocytes are near the tumor mass, but along the vessels), *around the tumor nest* (lymphocytes are around the tumor nests) and *focal* (lymphocytes are located focally, scattered).

Statistical analysis

In the second part of the research, for continuous variables such as Breslow thickness and mitotic rate, differences between groups were tested by Mann-Whitney U (comparing two groups) or Kruskal-Wallis tests (comparing three or more groups). Chi-square or Fisher's exact tests were applied to evaluate the association between categorical variables (e.g. patient groups, receptor expression, ulceration, melanoma localization, histological subtypes, TIL and PLI). To test the association between receptor expression and patient groups we used Chi-square test and a planned contrast approach.

Univariate and multivariate Cox proportional hazards model were applied to determine the impact of receptor expression, Breslow thickness, ulceration and pregnancy-associated characteristic of melanomas on DFS. Results are expressed as hazard ratios (HR) and 95% confidence intervals (CI). All statistic tests were two-sided and a $p<0.05$ was considered statistically significant. Statistical analyses were

carried out in IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.

4. Results

Clinicopathological characteristics of PAM patients

The majority of pregnancy associated melanomas arose *de novo* (27/35, 77,1%), while in one fifth of the cases (8/35 case, 22.9%) histologically confirmed that arise from preexisting naevus. The most common anatomical location in the PAM group was the lower limb (15/35; 42.8%), in NPAM, women group the trunk (14/32; 43.7%) and the lower limb (13/32; 40.6%), while in NPAM, men group the trunk (20/32; 62.5%). Most of the melanomas (23/34, 67.6%) were diagnosed in early stage, as in situ melanoma (2/34, 5.9%) or stage I (21/34, 61.7%). The most common histological subtype in all three groups was the superficial spreading melanoma (SSM), followed by nodular melanoma (NM). The mean Breslow thickness in PAM patients was 1.58±1.38 mm, the mean mitotic rate 4.74±6.04/mm^2. Most of the examined cases were Clark III (10/33, 30.3%) and Clark IV (12/33, 36.4%). The most common tumor cell type was the epitheloid, followed by the spindle cell type. There was no significant difference in the Bresow thickness, mitotic rate, tumor cell type, anatomical location, histological subtype and Clark level between the PAM and NPAM

groups. The presence of PLI was more common in PAM, than in NPAM, and the mild PLI was significantly more common in PAM than in NPAM (p=0.029) and NPAM women group (p=0.032). There was no significant difference found in the tumor ulceration, necrosis, regression or in the vascular invasion in the studied groups, and none of the patients had satellites. Five PAM patients developed one further tumor follwing the melanoma diagnosis. One patient presented simultaneously with two independent melanomas during pregnancy.

Serum S-100 protein level had been measured only in 25 PAM patients, which was in normal range at the time of diagnosis (0.052±0.02). BRAF mutation was analysed in 30 PAM patients, and in 11/30 cases (36.6%) the BRAFV600E was positive. Sentinel lymph node positivity was found in 7/34 PAM patients, while in 4/7 patients developed also metastases. As the follow-up time was rather short, the 5-year survival rate was evaluated only in 16 PAM patients, out of them 15 survived 5 years (93.75%).

Comparing the survival rate of PAM and the PAM patients by Kaplan-Meier analysis no significant difference was found. The mean survival in the PAM group was 122.29 (SD 6.60) months, in NPAM (women) 116.23 (SD 6.61) months and in NPAM (men) 91.70 (SD 4.19) months.

The frequency of the classical- and the non-classical estrogen receptors in the studied groups

All but one of the melanomas (1/81) failed to show any nuclear or cytoplasmic positivity for ERα. We could not find progesteron receptor (PGR) expression in the melanomas diagnosed during gravidity (0/20).

ERβ was expressed in all groups (during gravidity (PM): 85%, postpartum (PPM): 77.8%, PAM: 81.6%, NPAM-W: 59.1%, NPAM-M: 61.9%, NPAM: 60.5%). ERβ was more commonly expressed than GPER in NPAM patients (60.5% vs. 27.9% respectively, p= 0.0010). In the PAM group, both ERβ and GPER were expressed in a similar frequency (81.6% vs. 78.4%, p = 0.7290). No gender difference was found in the frequency of ERβ positivity among NPAM-M and NPAM-W or among PM and PPM.

GPER was present in the majority of PAM cases, while only in a subgroup of NPAM patients (78.4% vs. 27.9%; p = 0.0000). There was no significant difference found between the PM or PPM (85.0% vs. 70.6%, p=0.2886) melanomas, or between genders (22.7% vs. 33.3%, p= 0.4383). Thirty-nine of 41 (95%) GPER positive melanomas also co-expressed ERβ.

Double positive melanomas were significantly more common in PAM than in NPAM group (73.0% vs. 27.9%, p=0.0001). Similarly to ERβ and GPER, no gender difference was found in the frequency of

GPER/ERβ positivity among NPAM-M and NPAM-W (p = 0.4383) or among PM and PPM (p = 0.7633).

Concerning the frequency of GPER, ERβ or co-expression in pregnancy, no significant differences was found between the trimesters.

Association between the clinicopathological characteristics and the estrogen receptor expression

Among all ERβ-positive melanomas, the Breslow thickness was significantly lower than in ERβ-negative samples (mean 1.29 mm vs. mean 2.10 mm, p= 0.0093).

In GPER-positive cases, the Breslow thickness was lower, as compared to GPER-negative ones (mean 1.23 mm vs. mean 1.80 mm), but a significant difference was found only in NPAM-M group (mean 0.54 mm vs. mean 1.91 mm, p= 0.0256).

When GPER/ERβ double positive and double-negative melanomas were compared, the Breslow thickness was significantly lower in double-positive melanomas (mean 1.24, SD 1.03 vs. mean 2.23, SD 1.80) (p= 0.0156).

Considering melanomas as thin (≤1 mm) or thick (>1 mm), in all of the patients (n=81), both the soliter, and the simultaneous ERβ and GPER expression was significantly more common in thin (p=0.0032, p=0.0381 and p=0.0244) as in thick melanomas.

When all patients were examined, the mitotic rate was significantly lower in ERβ-positive, than in receptor-negative cases (mean 2.40, SD 2.71 vs. mean 6.00, SD 5.72) ($p = 0.0011$).

Cumulative data of all GPER-positive tumors also suggested a lower mitotic rate when compared to GPER-negative ones (mean 2.56, SD 2.72 vs. mean 4.57, SD 5.24, $p= 0.1027$), but a significant difference was reached only in NPAM-M (mean 0.50, SD 0.84 vs. mean 4.93, SD 5.28, $p= 0.0326$) and NPAM (mean 1.30, SD 1.57 vs. mean 4.90, SD 5.58, $p = 0.0260$).

Pooled data of GPER/ERβ positive melanomas showed a significantly lower mitotic rate compared to GPER/ERβ negative tumors (mean 2.35, SD 2.56 vs. mean 5.78, SD 5.82, $p = 0.0036$).

Tumor ulceration was rarely present among the patients and showed association only with GPER negativity among PAM ($p = 0.0489$).

The presence of PLI was significantly higher in ERβ, in GPER or in GPER/ERβ double positive melanomas than in receptor negative cases ($p= 0.0053$, $p= 0.0000$ and $p= 0.0001$, respectively).

In thin melanomas (mean Breslow thickness 0.89 mm) the leading edge PLI pattern was significantly more common ($p=0.0136$), while in thicker melanomas (mean Breslow 1.88 mm), the shoulder effect PLI pattern was more commonly present ($p=0.0104$). The PLI pattern was not associated with the presence of ER subtypes.

There was no significant difference found in the estrogen receptor status of *de novo* or naevus associated melanomas ($p=0.6601$, $p=0.9164$ and

p=0.9164), nor in the tumors in different anatomical localization (head-neck, upper extremity, trunk, lower extremity), nor in the histological subtypes (in situ, SSM, ALM, NCM or other) and the receptor expression.

The relation between estrogen receptor expression and the disease free survival

In univariate Cox proportional hazards model, the hazard of local or distant metastases was 0.696 lower (95% CI, 0.539-0.899) (p=0.0056), and in multivariate 0.785 lower (95% CI, 0.582-1.058) (p=0.1113) in GPER/ERβ double positive melanomas.

In thicker melanomas (>1 mm), the hazard of local and distant metastases was 7.912 times higher in univariate (95% CI, 2.769–22.604) (p= 0.0001) and 5.047 times higher in multivariate (95% CI, 0.529–48.148) (p= 0.1595) analysis compared to thinner melanomas (≤1 mm). The presence of ulceration significantly increased the hazard of local or distant metastases both in univariate (HR 3.515, 95% CI, 1.634–7.560) (p= 0.0013) and in multivariate (HR 5.638, 95% CI, 1.205–26.384) (p= 0.0281) analysis.

In multivariate analysis, pregnancy did not influence unfavorably the disease free survival of melanoma patients.

5. Conclusions

1) We examined the occurence of PAM among women in their reproductive age (18-45 years) in a Hungarian centre, and found a relatively high frequency of the tumor with a generally good clinical outcome, which can be explained by the early melanoma diagnosis and the favorable stage distribution.

2) During the comparison of the clinicopathological characteristics used in the routine diagnostic, we found no significant difference in the Breslow thickness, Clark level, mitotic rate, the presence of ulceration, satellites, vascular invasion, necrosis, regression between the PAM and the control NPAM patients in the same age and tumor stage. The peritumoral lymphocyte infiltration was more common in PAM than in the NPAM group.

3) The overall survival of PAM patients compared to NPAM patients in the same age and stage, during the examined study period, was not significantly different, similarly to the earlier publications.

4) In melanomas diagnosed during gravidity, progesteron receptor expression was not found. ERα was expressed only in one ALM case, therefore we suspect that these two receptors have evanescent role in the prognosis prediction in the majority of melanoma cases.

5) ERβ was expressed in all study groups with a high frequency, but it was significantly more common in PAM than in NPAM group (81.6% vs. 60.5%, p=0.0378). There was no significant difference found in the receptor expression between genders, or between melanomas during gravidity or postpartum.

6) The tumor thickness and the mitotic rate was significantly lower in ERβ positive tumor cells than in ERβ negative cases. The presence of PLI was significantly more common in ERβ positive than in ERβ negative melanomas, but there was no significant difference found neither in the presence/absence of ulceration, nor in the PLI pattern and in the ER expression.

7) We described the presence of GPER in human melanoma tissue. GPER expression was significantly more common in PAM than in NPAM men or women group. In NPAM group, GPER was less common than the ERβ. Similarly to ERβ, there was no significant difference found in the distribution of the receptor in NPAM group between genders, or between melanomas during gravidity or postpartum.

8) Similarly to ERβ, the Breslow thickness and the mitotic rate was significantly lower in GPER positive cases, moreover in PAM group, the presence of ulceration was significantly lower than in GPER

negative cases.

9) In most of GPER positive melanomas (39/41, 95%) ERβ and GPER was expressed simultaneously. The Breslow thickness and the mitotic rate was significantly lower in GPER/ERβ double positive melanomas than in double negative samples.

10) The hazard of local recurrences or metastases was lower in GPER/ERβ double positive melanomas. In cases of melanomas more than 1 mm and in cases of ulceration the hazard of local recurrences or metastases was higher. In multivariate analysis, pregnancy did not seem to influence unfavorably the prognosis of melanoma patients.

In summary, we described the presence of GPER in human melanomas and its co-expression with ERβ in the majority of PAMs and in a subset of NPAMs. GPER/ERβ double-positive melanomas had more favorable clinicopathological tumor characteristics and predicted a better clinical outcome. Therefore, our data suggest that the detection of solitary or simutaneous GPER and ERβ expression, may serve as new prognostic markers in localised melanoma.

6. Bibliography of the candidate's publications

Publications related to the thesis

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