Integrirani molekularni tkivni označevalci: matematični model v pristopu k oceni in predvidevanju raka prostate s tkivnimi molekularnimi označevalci

Molecular tissue markers integrated: A mathematical model approach to evaluating and predicting prostate cancer using molecular tissue markers

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Izvleček

Namen: *Standardni kriteriji za diagnozo raka prostate mnogokrat niso zadostni, da bi predvideli potek in izhod te pogoste bolezni. Vrednotenje molekularnih tkivnih označevalcev, še posebej tistih, ki označujejo apoptozo in proliferacijo, integrirani v matematični model, lahko pridonese k boljšemu razumevanju razvoja in patogeneze te bolezni; nadalje tudi k izboru oz. določitvi adekvatne terapije in bi odprlo tudi nove perspektive v iskanju novih terapij.*

Material in metode: *Parafinske tkivne rezine vzorcev prostatektomije smo imunohistokemično pobarvali po protokolih za p54, bcl-2 in CD105. Razvili smo matematični model, ki vključuje vrednosti tkivnih označevalcev glede na njihove apoptotično/proliferativne značilnosti.*

Abstract

Purpose: *The standard criteria available for the diagnosis of prostate cancer often do not sufficiently predict the course or outcome of this common disease. Evaluating molecular tissue markers, especially those that mark apoptosis and proliferation, in an integrated mathematical model, could contribute to a better understanding of the development and pathogenesis of this disease, aid in the selection of adequate therapies, and open new avenues of therapeutic research.*

Materials and methods: *Paraffin-embedded prostatectomy specimens were stained according to an immunohistochemical protocol for p53, bcl-2, and CD105. A mathematical model was developed, which incorporated values of tissue markers according to their apoptotic/pro-*

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Rezultate izražanja tkivnih označevalcev v matematičnem modelu smo korelirali s kliničnimi podatki bolnikov.
Rezultati: *V študiji se izkazuje oz. ugotavlja, da vrednosti tkivnih označevalcev bolje korelirajo s kliničnimi podatki, kadar jih uporabimo v matematični formuli, kot pa če jih uporabimo neodvisno.*

liferative characteristics.
Results: *The results of tissue marker expression and mathematical modeling were correlated with patient clinical data. We showed that tissue marker values correlated better with clinical data when tissue markers values were incorporated in a mathematical model than when applied alone.*

INTRODUCTION

Prostate cancer (CaP) is the most frequent cancer and most common cause of cancer-related death in men in developed countries [1]. CaP is also the most frequent type of cancer in Slovenian men [2].
CaP is a slow-progressing disease with a development phase about 10 years [3], however, it can also be an aggressive and fatal disease [4]. Despite comprehension data in expert literature, the molecular basis of CaP is poorly understood, and thus, the detection of clinically important CaPs and the optimal treatment remains a subject of debate [5].
Crucial is the knowledge of CaPs biologic potential, because one cannot always provide a prognosis or adequate therapy based on the current disease stage. Some patients die of CaP, while others live with CaP for many years. Current criteria available for the diagnosis, prognosis, and therapy are based on the TNM cancer stage assessment [6], Gleason score (GS) from biopsy specimen [7], and serum PSA levels [8-10]. However, those criteria and other clinical diagnostic data often do not sufficiently predict the course and outcome of the disease [11, 12].
Disease progression often occurs after radical prostatectomy or radiation therapy, and metastatic CaPs are not curable [13]. Therefore, it is important to identify cancers that will metastasize and choose a multimodal therapy versus those that will not metastasize and require observation only. Some cancer tissue markers could aid in disease prognosis and therapy determination [14, 15]. Tissue markers in CaP are relatively poorly studied, and thus, are not used in routine clinical practice. Some markers are used only for diagnoses in unclear cases [16]. The possible predictive value of molecular tissue markers would thus increase the understanding of disease.
Tumor growth occurs due to an imbalance between cell growth (proliferation) and cell death (apoptosis), but mainly due to increased cell proliferation [17]. Knowledge of the proliferation/apoptosis ratio could explain tumor dynamics, which would aid in the prediction of aggressiveness and the prognosis of disease. Proliferation and apoptosis could be determined by different tissue markers.
Expression of p53, which is a pro-apoptotic factor in irreparably damaged cells, has been linked to patient clinical characteristics and was used to predict biochemical recurrence after radical prostatectomy [18-21]. Patients with negative or low p53 expression have a good long-term prognosis [15].
In tumor cells, the p53 gene is mainly mutated due to DNA damage [17]. Studies have shown that apoptosis (number of apoptotic cells) could be a prognostic factor, in addition to clinical parameters, such as the PSA value and GS [5, 22]. Apoptosis has also been connected to CaP progression and the probability of low survival [22]. Another marker of proliferation is AgNOR, a synonym for nucleolar organizing regions (NORs), which are emphasized in actively proliferating cells [23, 24] and can serve as indicators of differentiation of high/low malignant tumors [25], or as prognostic factors in other tumors [26, 27].
Bcl-2 is an antiapoptotic gene [28], and its expression in the prostate is connected to cellular proliferation and could be used as a predictive factor for CaP prognosis [29, 30]. Some studies also connected its expression with GS [18, 31, 32] and with patient survival after

radical prostatectomy [19].

As with all tumors, CaP requires new vessels (angiogenesis) to expand, which can be measured in terms of "microvessel density" (MVD) [30, 33] and has prognostic value [34-38]. CD105 (endoglin) is an endothelial marker that is specific for new small blood vessels, and is overexpressed in tumor angiogenesis, but not in old vessels [39-42].

Many studies have introduced these and other molecular tumor markers in CaP, but there have been no consistent results. Thus, no new stand-alone tissue markers have been moved into clinics for prostate carcinoma [15]. The question is: is there a way to integrate research findings and make them usable in clinical practice by making them more indicative? Some studies indicated that the combined use of tissue markers could give better prognostic results than using individual marker levels in isolation [18, 43, 44]. However, no studies propose a method to integrate tissue marker expressions for CaP prognosis.

The purpose of this study was to develop a new way to integrate the results of different molecular markers expressed in CaP, and generate a general mathematical principle. With the introduction of a mathematical model, we will show the relations between the integrated tissue markers, with the purpose of defining connections that are not evident in the analysis of a single tissue marker (p53, bcl-2, CD105). We suggest that the integrated tissue markers used in the mathematical model will improve the prognosis of CaP and aid the choice of therapies.

MATERIALS AND METHODS

Preliminary study

Based on the idea of a mathematical model, a preliminary study was conducted [20]. Needle prostate biopsies from randomly selected patients were collected and analyzed using classic histology, histochemistry, and immunohistochemistry. The expression of AgNOR, p53, and bcl-2 was determined and correlated with patient clinical data (age, PSA level, GS before operation (on needle punction), presence of advanced metastatic disease, and AJCC status). A first mathematical model was then introduced and the data for the model were also correlated with patient clinical data.

Present study

This retrospective study was approved by the National Medical Ethics Committee of the Republic of Slovenia (No. 147/07/09). The study of separate molecular markers was performed between 2009 – 2018 by Mihael Munda at the Laboratory for Histology, Medical Faculty, University of Maribor.

Clinical data were obtained from Surgical Clinics in the Department of Urology, University Medical Centre of Maribor and from the archives of the Department of Pathology, University Medical Centre of Maribor. Fifty-five patients with a CaP diagnosis and prostatectomy were randomly selected for the study.

Clinical data that were collected included: patient's age at operation, PSA value (before operation), GS after operation (on prostatectomy specimen), T stage at diagnosis, and D'Amico score [45, 46].

As a basic approach, we assumed that the largest differences should be observed between classical and well-defined apoptosis/proliferation markers. Thus, p53 was selected as a common apoptotic marker, bcl-2 was selected as an antiapoptotic marker, and CD105 was selected as an proliferative marker.

Immunohistochemistry

Paraffin embedded tissue blocks were cut into 4 μm sections and stained according to an immunohistochemical protocol. Antigen recovery was achieved by heating the slides in an autoclave with sodium citrate buffer for 25 min. Endogenous peroxidase was inhibited with a Peroxidase Blocking Kit (Novocastra Laboratories, Newcastle upon Tyne, UK). For p53 protein staining, we used the dissolved lyophilized mouse monoclonal primary antibody, p53 DO-7 (1:50 dilution, Novocastra, Leica Biosystems, Newcastle Ltd., UK), which reacts with wild type and mutated p53. As positive and negative controls, breast adenocarcinoma and intestinal carcinoma were used.

For the detection of bcl-2, we used lyophilized the mouse monoclonal antibody, bcl-2 Oncoprotein (1:80 dilution, Novocastra, Leica Biosystems, Newcastle Ltd, UK). Sections of tonsil tissue were used as the positive control.

CD105 was detected using monoclonal mouse CD105 antibodies (1:50 dilution, Novocastra Laboratories, Newcastle Ltd, UK). Sections of tonsil tissue were

used as the positive control. Tissue sections were counterstained using Mayer's hematoxylin and mounted.

For the determination of p53 and bcl-2 expression, whole specimens were examined at 100x magnification and three areas of morphometric evaluation were defined (hot spots) [47]. The hot spots were the areas with the most dense and intense staining. At 400x magnification, we counted all positive and negative stained glandular cells in a view-field of each hot spot. The results of staining analyses are expressed as a p53 and bcl-2 index value that represents the number of positive stained cells out of 1,000 counted cells.

For the determination of CD105 expression, three areas of maximal angiogenesis (hot spots) within the tumor were identified. Then, micro vessels were counted at 400x magnification in each area. Any single cell or spot stained by the immunohistochemical marker was counted as a vessel [35, 38]. Then, the mean vascular count per mm2 was calculated and expressed as mean vascular density (MVD).

Mathematical model

A mathematical model was developed to evaluate the staining for apoptotic (p53) and proliferative (Bcl-2 and CD105) markers, and relate it to clinical data. The model was designed with the consideration that the expression of apoptotic and proliferative tissue markers should oppose each other.

This mathematical model was expressed as:

index p53 - index bcl-2 – MVD CD105.

The model was applied to the data for each patient separately. A more negative value was indicative of increased cellular proliferation and a more positive value (or closer towards zero) was indicative of a reduction in proliferation.

Data analysis

Correlations between clinical data and the three individual tissue markers, as well as between clinical data and the output of the mathematical model were analysed using a two-tailed Spearman (rho) correlation coefficient. A $P < 0.05$ was considered statistically significant. Data were analyzed using IBM SPSS Statistics 19.0 (IBM-SPSS, Chicago, IL, USA).

RESULTS

P53 was observed in cell nuclei (Fig. 1) and bcl-2 was observed in the cytoplasm and on the nuclear membrane of glandular cells (Fig. 2). CD105 was expressed in endothelial cells of newly formed vessels. P53 expression was evident in 95% of specimens. Bcl-2 and CD105 expression was evident in all (100%) specimens.

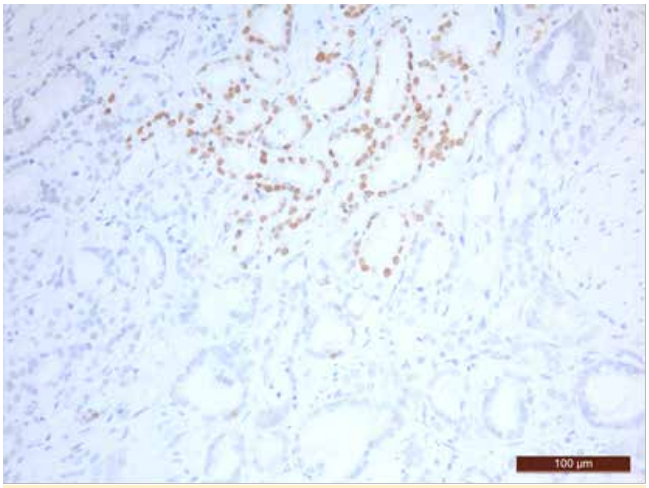

Figure 1. *Expression of p53 tissue marker*

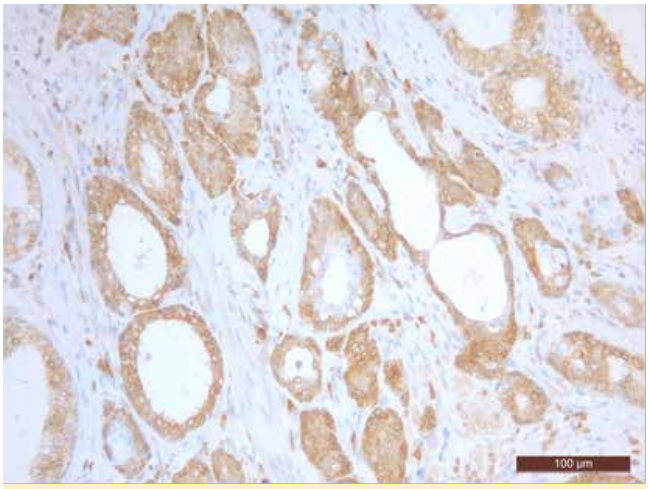

Figure 2. *Expression of bcl-2 tissue marker*

Index p53 was significantly positively correlated with GS after operation ($r = 0.56$, $P < 0.001$). Index bcl-2 was significantly negatively correlated with GS after

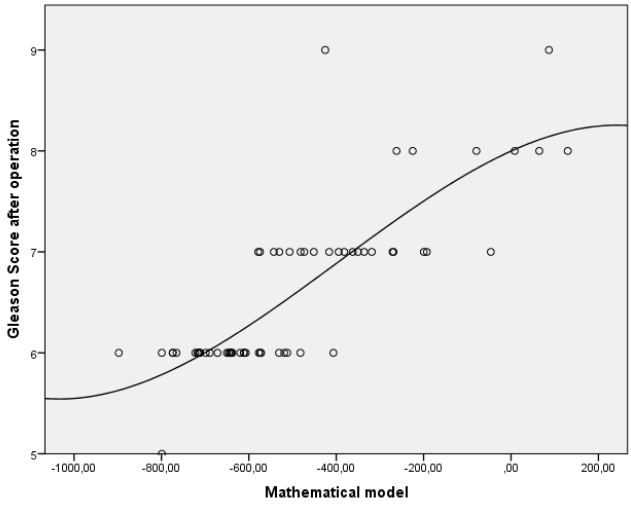

Figure 3. *Correlation of mathematical model*

operation (r = – 0.78, P < 0.001), and also negatively correlated with T stage (r = – 0.37, P = 0.005) and with D'Amico score (r = – 0.25, P = 0.048). MVD CD105 was significantly positively correlated with patient age (r = 0.315, P = 0.014) and with T stage (r = 0.36, P = 0.006). The mathematical model was statisticaly significantly positively correlated with GS after operation (r = 0.84, P < 0.001) (Fig. 3), with T stage (r = 0.29, P = 0.029) and with D'Amico score (r = 0.31, P = 0.017). Table 1 summarizes the correlations between clinical parameters, indexes, and the mathematical model.

DISCUSSION

As a single marker, index bcl-2 showed the strongest correlations with clinical data, while MVD CD105 and index p53 had fewer correlations. Some specific differences in the correlations between tissue marker expression and GS and T stage also occurred. One would not expect that there would be a strong correlation between index p53 and GS, but no correlation with T stage, and exactly the opposite situation with MVD CD105. The situation becomes interesting when there are correlations between index bcl-2 and GS, and between index bcl-2 and T stage, and between index bcl-2 and D'Amico score. The conclusion should be that bcl-2 is the best stand-alone marker for prostate cancer. The mathematical model correlated with the same clinical data as index bcl-2, and therefore, the conclusion would be again that the bcl-2 is the most important marker, or that index bcl-2 is the strongest variable in the model. However, there is much more than just this narrow sight. When we analyze the data closely, we see two unexpected phenomena:

1. The correlation between the mathematical model and GS is higher than the individual correlations between index p53 and GS, or between index bcl-2 and GS.
2. The mathematical model correlates with T stage and D'Amico score, although to a slightly lower extent than index bcl-2 did, regardless the fact that index p53 and MVD CD105 did not correlate with either of those clinical parameters.

Thus, index p53 and MVD CD105 are contributing to the mathematical model in a negative way, and the correlation of the mathematical model with T stage and D'Amico score was lower than the correlation of index bcl-2 with both of them. Conversely, an unknown factor, which we assume to be index p53, increased the model correlation with GS in a positive way, despite that no single tissue marker has such a high correlation with GS. The importance and the mechanism of this finding will be the topic of a subsequent study.

The results of this study agree with the results of our

Table 1: *Correlations between tissue markers, the mathematical model, and clinical data.*

	Patient's age	GS	T stage	D'Amico score
Index p53	r = 0.56, P < 0.001	NS	NS	NS
Index bcl-2	NS	r = – 0.78, P < 0.001	r = – 0.37, P = 0.005	r = – 0.25, P = 0.048
MVD CD105	r = 0.315, P = 0.014	NS	r = 0.36, P = 0.006	NS
Mathematical model	NS	r = 0.84, P < 0.001	r = 0.29, P = 0.029	r = 0.31, P = 0.017

GS = Gleason Score, NS = not significant

preliminary study [20]. In both studies that included different molecular markers (AgNOR vs CD105 MVD), we confirmed that tissue markers integrated in a mathematical model contributed to better correlations with clinical data and more consistent results than tissue markers alone.

Both mathematical models differed in the tissue markers that were used (AgNOR in previous study vs. CD105 in present study), and in the usage of logarithmic data conversion, which was applied only in the preliminary study [20] because of the small number of patients included (17 patients). In present study, no logarithmic conversion was used as more (55) patients were included.

The outcomes of both models were based on the potency of apoptosis versus the potency of proliferation. Both models also showed increasingly negative (or lower) values as proliferation increased, and less negative (or higher) values when proliferation was reduced. Also, both models showed that when the values of immunohistochemical expression were integrated in the mathematical model, the correlation to the clinical data was more significant than when immunohistochemical expression was used alone.

CONCLUSIONS

These data lead us to conclude that there are important connections and interactions between the expression of molecular tissue markers in CaP and that they should be taken into consideration as they can affect study results.

We confirmed that statement in two similar studies. Thus, there is an indication that when dealing with tissue markers in CaP, we should use integrative-mathematical approaches that take multiple variables into consideration, which could have prognostic value for prostate cancer. Additional research with a larger number of patients and clinical data should be performed to determine the optimal variables and to define an optimal model for use in clinical practice.

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CONFLICT OF INTEREST

The authors of this study declare that they have no conflict of interest related to the research.

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