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Original Research Article

Cell-cycle gene expression analysis using real time PCR in locally advanced squamous-cell head and neck cancer

Grzegorz Woźniak [a,*], Robert Herok [b], Roman Jaksik [c], Maciej Misiołek [d], Bogdan Kolebacz [e], Anna Fiszer-Kierzkowska [b], Katarzyna Miśkiewicz-Orczyk [d], Cezary Szymczyk [f], Adam Maciejewski [f], Grzegorz Głowacki [a], Rafał Suwiński [g]

[a] Radiotherapy Department, Maria Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice Branch, Gliwice, Poland
[b] Center for Translational Research and Molecular Biology of Cancer, Maria Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice Branch, Gliwice, Poland
[c] Institute of Automatic Control, Silesian University of Technology, Gliwice, Poland
[d] Clinical Department of Otolaryngology in Zabrze, Medical University of Silesia, Katowice, Poland
[e] Department of Laryngology, Upper Silesian Medical Center, Katowice-Ochojec, Poland
[f] Clinic of Oncological and Reconstructive Surgery, Maria Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice Branch, Gliwice, Poland
[g] II Radiotherapy and Chemotherapy Clinic and Teaching Hospital, Maria Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice Branch, Gliwice, Poland

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ABSTRACT

Purpose: The analysis of gene expression, especially those involved in cell cycle control, can help to discover mechanisms determining the outcome of radiation treatment. The main purpose of this study was to examine the expression level of genes responsible for cell cycle regulation in samples of the head and neck cancer, obtained during surgery.
Methods: Postsurgical samples of SCC of head and neck region were collected. Over 80 genes were analysed using cell cycle quantitative real-time RT-PCR Array method. Presence of 14 high-risk HPV types DNA in frozen or paraffin-embedded tumour pathological samples was also assessed. To correlate gene expression with selected pathological features and clinical outcome we used different hierarchical clustering method.
Results: Hierarchical clustering demonstrated the association between gene expression within certain clusters and gender, tumour site, T stage, N stage, grade, pathological subtype or tumour recurrence.
Conclusions: Despite some limitations we were able to identify gene clusters that allowed to classify patients according to selected clinical features and occurrence of tumour recurrence. The results of the analysis also confirm that the incidence of HPV infection among the patients from Upper Silesia is relatively low, whereas HPV negative tumours, likely associated with smoking, appeared dominant.
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1. Introduction

Head and neck cancers share about 5% of all malignancies in Poland [1]. Combined therapy is used in locally advanced cases with the dominant role of surgery. Personalised diagnostic and therapeutic process have been the mainstay of the clinical development throughout the last decade [2]. Development of clinically useful molecular markers, which could be derived from tumour samples during a surgery, is considered to be the way forward in modern research. Such markers may possibly allow for optimal selection of patients to tailor then diverse treatment modalities and help optimisation of the treatment sequence and its intensity. Evaluation of those processes and understanding of their effect upon the cell cycle regulation and, consequently, the treatment response have now become the major challenges of oncology.

The main aim of this study was to examine the expression level of genes responsible for cell cycle regulation in samples of the head

* Corresponding author at: Radiotherapy Department, Maria Sklodowska-Curie Memorial Cancer Center and Institute of Oncology, Gliwice Branch, Wybrzeże Armii Krajowej 15, 44-100 Gliwice, Poland. Tel.: +48 32 278 8001; fax: +48 32 278 8001.
E-mail address: gwozniak.md@gmail.com (G. Woźniak).

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and neck cancer, obtained during surgery. Expression of the cell cycle genes may affect the proliferation rate of tumour cells as well as the repopulation rate (i.e. re-growth during and after treatment), consequently appearing as a potential prognostic and/or predictive factor in postoperative radiotherapy or radio-chemotherapy. We have previously performed a large sample study on histochemically assessed expression of selected genes as prognostic and predictive markers in postoperative radiotherapy for head and neck cancer [2]. Here we seek the potential new markers to be further studied in a larger cohort of the patients.

2. Materials and methods

2.1. Materials

The selection criteria included locally advanced cancer of the head and neck, the preoperative assessment of squamous cell cancer, treatment, comprising the major surgery, as well as the informed consent of the patients to participate in the study. Only those patients who were pre-operatively considered as potential candidates for post-operative radiotherapy or radio-chemotherapy were included. The protocol of the study, included the patient's informed consent form and the approval by the local Bioethical Committee, as demanded by the national regulations. The study group comprised the patients who received treatment between 2008 and 2011, at the Medical University of Silesia and/or Maria Skłodowska-Curie Memorial Cancer Centre in Gliwice.

Tumour tissue specimens from 21 patients were collected upon surgery and placed in the RNAlater solution for further evaluation. 17 out of the 21 samples (80.9%) showed the RNA quality sufficient for analysis of gene expression. In the remaining 4 patients, isolation of RNA did not appear feasible due to necrotic tumours or insufficient amount of the tumour cells available.

The age of the resulting 17 patients included in the analysis ranged from 45 to 80 years (median 58) with a gender split of 8 males and 9 females. The most frequent tumour location was the tongue (7 instances), the retromolar triangle and the mouth floor (3 instances), the tonsils (2 instances) and the larynx (1 instance). Eight patients were post-operatively staged as T2, five as T4 and three as T3. Nine patients showed the microscopically confirmed nodal metastases. The preoperative diagnosis of squamous cell carcinoma was confirmed in all patients. Pathological Grade 2 was established in 8 cases, G1 in 4 cases, G3 in 1 case, while in the remaining 4 cases the grade was not assessed. The clinical characteristic of the group is presented in Table 1. All 17 patients included received the postoperative radiotherapy.

2.2. Total RNA isolation

Up to 5 μg piece was homogenised with the use of FastPrep 24 homogeniser (MP Biomedicals), tubes with Lysis Matrix D (MP Biomedicals), 2× 30 s, 6000 rpm. Total RNA was isolated using RNeasy Micro Kit (74 004 – Qiagen) and RNase-Free DNase Set (79254 – Qiagen) following the manufacturers' instructions. For the lysate homogenised in 600 μl buffer RLT (with β-mercaptoethanol) 600 μl 70% EtOH/DEPC was added and mixed thoroughly. The entire volume was then fed to the column and centrifuged for 30 s, 12,000 × *g* (14,000 rpm). Next, 350 μl of RW1 buffer was added and the mixture was centrifuged for 30 s at 12,000 × *g* (14,000 rpm). 70 μl of RDD Buffer was added to the 10 μl of DNase carefully, and the whole volume (80 μl) was applied exactly to the middle of the column. After 15 min incubation at room temperature, 350 μl of RW1 buffer was added and centrifuged for 30 s at 12,000 × *g* (14,000 rpm). The column was then placed in a new tube. 500 μl RPE buffer was applied to the column and centrifuged for 30 s at 12,000 × *g* (14,000 rpm). The filtrate was removed and 500 μl RPE buffer was re-applied and centrifuged for 2 min at 12,000 × *g* (14,000 rpm). The mixture was then transferred to a new column [1.5 mL], 25 μl H_2O "RNase-free" was added and centrifuged for 1 min at 12,000 × *g* (14,000 rpm). Following that, 20 μl H_2O "RNase-free" was added and centrifuged for 1 min at 12,000 × *g* (14,000 rpm). The purity of RNA samples, assessed with a spectrophotometer, was between 1.9 and 2.0. The RNA samples were also analysed with the 2100 Bioanalyzer (Agilent). The values of RNA Integrity Number (RIN) were between 6.5 and 8.6.

2.3. Quantitative reverse transcription polymerase chain reaction (QRT-PCR)

cDNA was synthesised and used it in the Q-PCR reaction, based on RT Profiler PCR Array System from SABiosciences (Human Cell Cycle RT2 Profiler PCR Array 3.0, PAHs-020D-12, RT2 Real-Time PCR SYBR Green Master Mix, PA-010-12, RT2 PCR Array First Stand Synthesis Kit, C-03). The kit contains probes for the expression level assessment of more than 80 genes involved in the cell cycle, including: G1 phase and the G1/S transition (ANAPC2, CCND1, CCNE1, CDC34, CDK4, CDK6, CDKN1B, CDKN3, CUL1, CUL2, CUL3, SKP2), S phase and DNA replication (ABL1, MCM2, MCM3, MCM4, MCM5, PCNA, RPA3, SUMO1, UBE1), G2 phase and the G2/M transition (ANAPC2, ANAPC4, DIRAS3, BCCIP, BIRC5, CCNB1, CCNG1, CCNH, CCNT1, CCNT2, CDK5R1, CDK5RAP1, CDK7, CDKN3, CKS1B, CKS2, DDX11, DNM2, GTF2H1, GTSE1, HERC5, KPNA2,

Table 1
Clinical characteristics of the group.

ID	Age	Sex	Tumour location	T stage	N stage	HP subtype[a]	Grading	Number of positive lymph nodes
5	45	M	Floor of the mouth	2	0	1	1	0
6	56	M	Floor of the mouth	1	0	1	1	0
15	56	F	Floor of the mouth	2	2	2	2	3
1	62	M	Larynx	4	2	2	2	3
19s	80	F	Retromolar triangle	4	0	1	3	0
25s	53	F	Retromolar triangle	3	1	N	2	3
28s	59	F	Retromolar triangle	3	2	1	2	3
3	57	F	Tongue	3	2	1	2	3
7	61	M	Tongue	2	1	1	N	1
16	60	F	Tongue	2	0	N	N	0
19	68	M	Tongue	2	1	N	1	1
21	54	M	Tongue	2	1	1	2	1
24	65	M	Tongue	2	0	N	2	0
24s	73	F	Tongue	2	0	N	2	0
25	53	F	Base of tongue	4	0	2	1	0
18	54	M	Tonsil	4	2	2	N	3
20	75	M	Tonsil	4	0	2	N	0

[a] Histopathological subtypes – (1) Squamous cell ca; (2) Partial squamous or non-squamous cell ca; N. unknown.

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