Gene Expression Profiling of Circulating Natural Killer Cells in Head and Neck Squamous Cell Carcinoma

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Abstract. *Background: Natural killer (NK) cells are a major player of the innate immune system. Besides known risk factors for head and neck squamous cell carcinoma (HNSCC), impaired immune surveillance may as well lead to tumor manifestation. Materials and Methods: In the present study, microarrays technologies were employed in order to perform gene expression profiling of NK cells in HNSCC patients. Differentially expressed genes have been detected in a comparative setting consisting of non-treated HNSCC patients, treated recurrence-free HNSCC patients and healthy control subjects. Results: Cytokine receptors such as CCR7, IL-7R, and CXCR3 were down-regulated in non-treated HNSCC patients compared to healthy subjects, indicating impaired immune surveillance. In treated patients, up-regulated genes such as receptors for Fc fragments of specific antibodies, or ficolin-1 can be withheld as an immunological response to tumor manifestation. Conclusion: This study provides insights into gene expression changes in NK cells of HNSCC dependent upon clinical status and introduces several candidate genes suitable for further investigation.*

Preliminary results of this study have been presented by the corresponding author at the 80th Annual Meeting of the German Society of Oto-Rhino-Laryngology, Head and Neck Surgery, May 20-24, 2009 in Rostock, Germany.

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Key Words: Natural killer cells, cytokine receptors, gene expression, HNSCC, immune surveillance, gene microarrays.

Head and neck squamous cell carcinoma (HNSCC) is the sixth leading cancer worldwide with an estimated incidence of 600,000 cases annually (1). The moderate prognosis of an approximately 40-50% mean 5-year survival has sadly remained unchanged for decades even though advances have been made in the understanding of tumor biology and individualized therapeutic schemes have been established (2, 3). Well-known risk factors for developing HNSCC are tobacco smoke, especially in combination with alcohol abuse, poor oral hygiene, Epstein–Barr virus (EBV) and, particularly in oropharyngeal carcinoma, human papillomavirus (HPV) infection. Other exogenous noxae such as ionizing radiation, hardwood dust, asbestos and chewing of betel nuts are also thought to play a role in carcinogenesis (4).

Progress in understanding the immune system and its functions has put emphasis on possible impaired immunological surveillance as one reason for tumorigenesis and progression (5, 6). The interactions of tumor cells and the immune system are highly complex and they are subject to a variety of control circuits of partly cytokine-mediated, cellular (T-lymphocytes, natural killer (NK) cells, dendritic cells (DCs), eosinophils, macrophages) and humoral (mainly plasma cells) mechanisms (7, 8). Elimination of malignantly transformed cells is mainly restricted to T-lymphocytes (cytotoxic $CD8^+$ T cells), DCs and NK cells (9, 10). Besides this group of cells, regulatory function in anti-tumor immunity for HNSCC is assumed to be provided by $CD4^+$ sub-groups such as T_H17 and T-regulatory cells (Tregs), NKT cells, and γδT cells (11).

NK cells are part of the innate cellular immune system and can be described as large granular lymphocytes. Following the cluster of differentiation (CD) nomenclature NK cells can be characterized as $CD3^-$, $CD16^+$, $CD56^+$ and they represent 6-29% of peripheral blood mononuclear cells

Table I. *Probands' basic clinical characteristics*

Clinical group	Gender	Age (years)	Tumor site	UICC-Stage (acc. Ref 21)	Recurrence-free period after therapy in months, (type of therapy)	NK proportion in PBMC (%)
Non-treated	♀	51	Larynx	IV	-	13.47
Non-treated	♂	45	Larynx	IV	-	16.42
Non-treated	♂	67	Oropharynx	II	-	12.31
Non-treated	♂	55	Oropharynx	IV	-	13.21
Treated	♂	66	Oropharynx	IV	14 (S, RT)	25.84
Treated	♂	63	Hypopharynx	IV	12 (S, RT)	31.19
Treated	♂	44	Hypopharynx	I	108 (S, RT)	25.06
Treated	♂	53	Oropharynx	II	60 (S, RT)	25.56
Healthy	♂	40	-	-	-	15.25
Healthy	♂	18	-	-	-	13.78
Healthy	♀	48	-	-	-	15.99
Healthy	♀	51	-	-	-	13.48

S: Surgery; RT: adjuvant radiotherapy.

(PBMC) in healthy individuals (12). On the basis of surface molecule expression, NK cells are divided into 'CD16^{+} CD56^{dim}' and 'CD16^{-} CD56^{bright}'. The first of these, representing 90-95% of all mature NK cells, mainly circulate in the periphery and are characterized by low cytokine production and high cytotoxicity (13, 14). In contrast to T-cells, NK cells are not subject to major histocompatibility complex (MHC) restriction and are capable of mediating cytolytic reactions without prior sensitization in virus-infected or malignantly-transformed cells in different ways (15). NK cells recognize target cells by reduced or absence of autologous MHC-I-expression ('missing-self' hypothesis) (16), disabling NK receptors (killer-cell immunoglobulin-like receptors (KIRs) and CD94/NKG2A receptors) to initiate cytolysis-inhibiting signal cascades. The natural cytotoxicity of NK cells is mediated by the perforin/granzyme mechanism. Other apoptosis-inducing mechanisms such as antibody-dependent cellular cytotoxicity (ADCC), Fas ligand (FasL), tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) activation or TNF-α secretion have been recently discovered (17, 18). In addition to direct cytotoxicity, NK cells play an important role in the regulation of the anti-tumorous adaptive immune response as they produce cytokines such as interferon-γ (IFN-γ), TNF-α, interleukin (IL)-10, several chemokines, and growth factors. Thus, NK cells exert an influence on macrophages, neutrophils and DCs during the immune response (19).

Earlier results of our group revealed significantly increased proportions of circulating NK cells in PBMC in treated recurrence-free HNSCC patients (26.39±11.52%, $p<0.001$) compared to non-treated HNSCC patients (15.47±7.31%) and healthy individuals (15.41±6.87%) (20). In order to understand these changes in distribution and obtain explanations for the clinical status at the genomic level (recurrence-free or manifested HNSCC), we performed a comparative gene expression analysis of NK cells from patients belonging in different clinical groups.

Materials and Methods

Patients. The present study was conducted in accordance with the revised version of the Helsinki Declaration. The study design was approved by the Ethics Committee of the General Medical Council of Mecklenburg Western Pomerania. All patients gave their written consent.

From an initial cohort of 105 probands, we investigated a total of 12 representative subjects who were divided into three clinical groups: "Healthy", healthy control subjects; "Non-treated" patients with HNSCC and no therapy up to that point; "Treated", patients, no evidence of locoregional recurrence (relapse-free ≥12 months) after HNSCC.

All study participants were at least 18 years old and had no underlying malignant, hematological or autoimmune disease. All tumor patients in the groups 'Non-treated' and 'Treated' (n=8) had histologically confirmed HNSCC with certain degrees of differentiation (G1, n=0; G2, n=7; G3, n=1; G4, n=0). Tumor staging including regional and distant metastases was carried out according to the 7th edition of the TNM classification by the International Union Against Cancer (UICC) 2010 (21).

In the 'Treated' group, the mean relapse-free period after therapy until venous puncture was 48.5 months (range from 12 to 108 months). The 'Treated' patients had a mean of 26.91% NK cells in PBMC and a mean age of 56.5 years, whereas 'Non-treated' patients on average were 54.5 years old and had a mean of 13.85% NK cells in PBMC. The group 'Healthy' had a mean age of 39.3 years and a mean of 14.63% NK cells in PBMC. The probands' basic clinical parameters are presented in Table I.

Cell depletion and counting. Peripheral venous blood (37.5 ml) was obtained under sterile conditions on Wednesdays between 9 a.m. and

12 a.m. The samples were collected in EDTA blood collection tubes (Serum-Monovette 7.5 ml K3E, 1.6 mg EDTA/ml; Blut, Sarstedt, Nümbrecht, Germany). Then isolation of PBMC by density-gradient centrifugation was carried out over a Ficoll-Paque Plus (Amersham Bioscience, Buckinghamshire, UK) gradient according to the manufacturer's instructions. After addition of phosphate-buffered saline (PBS) tablets, (Sigma–Aldrich, Seelze, Germany) and centrifugation, the PBMC were isolated. Following antibody conjugation (CD3 MicroBeads and CD56 MicroBeads, Miltenyi Biotec, Bergisch Gladbach, Germany), lymphocytes were separated by immunomagnetic cell depletion, using magnetic activated cell sorting (MACS) technology, into $CD3^+$ (T-cells) and $CD3^-/CD56^+$ (NK cells) that were used for further immunological investigations.

PBMC were then labeled with fluorochrome-conjugated antibodies (Simultest CD3/CD16+CD56 Reagenz; Becton Dickinson, San Jose, CA, USA). Afterwards, the cells were counted and characterized in terms of size, granularity and maximum emission of the selectively conjugated dyes phycoerythrin ($CD16^+$, $CD56^+$) and fluorescein isothiocyanate ($CD3^+$) by flow cytometry (FACSCalibur Flow Cytometer; Becton Dickinson). FACS (fluorescent activated cell sorting) immunophenotyping enables for counting and differentiation of NK cells and T cells.

RNA extraction, labeling, and hybridization of microarray targets. Following the standard operating procedure of the Institute of Immunology, University of Rostock, the separated NK cell specimens were subjected to total RNA extraction combined with simultaneous removal of genomic DNA using the RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA integrity and purity were verified by electrophoresis on agarose gel and by spectrophotometry (Spectralphotometer ND-1000, NanoDrop Technologies, Wilmington, DE, USA). RNA probes were then labeled and hybridized using 2.5 μg of total RNA, according to the supplier's instruction ("GeneChip® Expression 3' Amplification One-Cycle Target Labeling and Control reagents", Affymetrix, St. Clara, CA, USA).

In detail, the first-strand cDNA was synthesized using 2.5 μg of whole RNA sample and SuperScript II Reverse Transcriptase (RNaseH minus) introducing a T7-$(dT)_{24}$ primer. The second strand synthesis was performed as a strand replacement reaction using the *E. coli* DNA-Polymerase I complex, hybrid strand specific RNA degrading RNaseH, a ligase reaction (*E. coli* DNA Ligase) and finally an end-polishing with recombinant T4-Polymerase was performed. Biotin-16-UTP was introduced as a label by a linear-amplifying in vitro transcription reaction using T7 polymerase overnight (16 h). The required amount of cRNA was fragmented by controlled chemical hydrolysis to release the proportion of cRNA molecule length and the amount of incorporated biotin derivative. Hybridization was carried out overnight (16 h) at 45°C in a GeneChip® Hybridization Oven 640 (Affymetrix). Subsequently, washing and staining protocols were performed with the Affymetrix Fluidics Station 450. For signal enhancement, antibody amplification was carried out using a biotinylated anti-streptavidin antibody (Vector Laboratories, Peterborough, UK), which was cross-linked with goat IgG (Sigma, Seelze, Germany), followed by a secondary staining with a streptavidin-phycoerythrin conjugate (Molecular Probes, Invitrogen, Karlsruhe, Germany). Scanning of the microarray was performed with a GeneChip® Scanner 3000 (Affymetrix) at 1.56-micron resolution. Data analysis was performed with MAS 5.0 (Microarray Suite statistical algorithm, Affymetrix). The probe expression level analysis was carried out using GeneChip® Operating Software (GCOS 1.4) and the final data extraction was done with the DataMining Tool 3.1 (Affymetrix).

Microarray chip. Gene expression analysis was carried out using the GeneChip® Human Genome U133 plus 2.0 (Affymetrix). This microarray technique analyzes the expression levels of over 47,000 transcripts and variants including 38,500 well-characterized human genes.

Comparative setting. To identify differentially expressed genes, which may act as candidates to clarify the changes in distribution of circulating NK cells, we created three comparative groups each consisting of two clinical groups with four subjects each. "Treated *vs*. Non-treated", "Non-treated *vs*. Healthy" and "Treated *vs*. Healthy".

Each comparative group yields relative gene expression levels based on the whole human genome of each of the four clinical group members (experimental array) versus each member of the other group (baseline array). This leads to 16 cross-comparisons (4×4) for each probe set (unit on the GeneChip®, representative of one gene) in each comparative group.

Statistical analysis. Data analysis and employed statistical algorithms were carried out following the manufacturer's instructions and guidelines (GeneChip® Expression Analysis, Data Analysis Fundamentals; www.affymetrix.com). The arbitrarily set cut-offs for candidate probe sets were defined as having a detection call 'present' in at least 15 of 16 cross-comparisons and a mean fold change value of ≥2.0 when comparing the experimental array to the baseline array.

Functional interpretation. All detected probe sets/differentially expressed genes were analyzed *via* the web portal Netaffx™ Analysis Center (Affymetrix; http://www.affymetrix.com/analysis/index.affx) and the linked bioinformatics initiative Gene Ontology (GO). Additionally, selective MEDLINE database searches concerning candidate genes were performed *via* PubMed (last accessed on 12 April 2013).

Results

Comparison of 'Treated vs. Non-treated' group. In this comparative group, altogether nine differentially expressed genes could be detected within the inclusion criteria. In comparison to the baseline array 'Non-Treated', six down-regulated genes could be identified in the experimental array 'Treated'. Amongst others, an 8.5-fold relative underexpression of *PROK2*, whose gene product is involved in chemotaxis, became evident in the NK cells of 'Treated' patients (Table II). Furthermore, three up-regulated genes, *e.g. KLRC3*, which is associated with the cellular defense response, could be detected in 'Treated' HNSCC patients (Table III).

Comparison of 'Non-treated vs. Healthy' group. When comparing probe sets in this comparative group, differences in expression levels of 47 genes were revealed. Within the

Table II. *Differentially expressed genes in group comparison 'Treated vs. Non-treated'. Change Call: Decrease.*

Probe set ID	Gene symbol	Gene title	GO biological process term	Cytoband location	Mean fold change
232629_at	*PROK2*	Prokineticin-2	Chemotaxis, cell prolifferaion	3p13	–8,46
222670_s_at	*MAFB*	v-maf Musculoaponeurotic fibrosarcoma oncogene homolog B (avian)	Regulation of transcription	20q11.2-q13.1	–6,29
209616_s_at	*CES1*	Carboxylesterase-1	Metabolic process, response to toxin	16q22.2	–5,09
224917_at	*MIRN21*	microRNA-21	–	17q23.1	–4,86
227697_at	*SOCS3*	Suppressor of cytokine signaling-3	Response to cytokine stimulus	17q25.3	–4,84
1569003_at	*TMEM49*	Transmembrane protein-49	-	17q23.1	–3,78

Table III. *Differentially expressed genes in group comparison 'Treated vs. Non-treated', Change Call: Increase.*

Probe set ID	Gene symbol	Gene title	GO biological process term	Cytoband location	Mean fold change
224215_s_at	*DLL1*	Delta-like 1 (Drosophila)	Regulation of cell adhesion	6q27	4,29
207723_s_at	*KLRC3*	Killer cell lectin-like receptor subfamily C, member 3	Cellular defense response	12p13	3,24
204995_at	*CDK5R1*	Cyclin-dependent kinase 5, regulatory subunit 1 (p35)	Cell proliferation, neuron migration	17q11.2	2,66

Table IV. *Differentially expressed genes in the 'Non-treated vs. Healthy' group of comparison. Change Call: Decrease.*

Probe set ID	Gene symbol	Gene title	GO biological process term	Cytoband location	Mean fold change
234013_at	*TRD@*	T-cell receptor delta locus	Immune response	14q11.2	–85,34
212730_at	*SYNM*	Synemin, intermediate filament protein	Cytoskeleton organization	15q26.3	–20,97
207979_s_at	*CD8B*	CD8b molecule	Immune response	2p12	–12,87
206337_at	*CCR7*	Chemokine (C-C motif) receptor-7	Chemotaxis, immune response	17q12-q21.2	–11,97
226218_at	*IL7R*	Interleukin-7 receptor	Immune response	5p13	–10,70
203413_at	*NELL2*	NEL-like-2 (chicken)	Cell adhesion, regulation of growth	12q12	–10,28
230489_at	*CD5*	CD5 molecule	Induction of apoptosis	11q13	–8,36
217147_s_at	*TRAT1*	T-cell receptor associated transmembrane adaptor-1	Cellular defense response	3q13	–5,95
207681_at	*CXCR3*	Chemokine (C-X-C motif) receptor-3	Chemotaxis	Xq13	–5,28
209570_s_at	*D4S234E*	DNA segment on chromosome 4 (unique) 234 expressed sequence	Dopamin receptor signaling pathway	4p16.3	–4,12
222392_x_at	*PERP*	PERP, TP53 apoptosis effector	Induction of apoptosis	6q24	–3,98
204642_at	*S1PR1*	sphingosine-1-phosphate receptor-1	Positive regulation of positive chemotaxis	1p21	–3,51
202746_at	*ITM2A*	Integral membrane protein 2A	–	Xq13.3-Xq21.2	–2,81

inclusion criteria, 13 genes were found down-regulated in the experimental array 'Non-treated' compared to 'Healthy'. Amongst others, 'Healthy' patients had an overexpression of chemokine receptors (*CCR7* and *CXCR3*), *IL-7* receptor and the apoptosis effector *PERP* (Table IV). 'Non-treated' probands showed an up-regulation of 34 genes including several gene products that could be attributed to immune response such as immunoglobulin receptors (*FCAR*, *FCGR1A* and *C*) or gene products for chemotaxis (*CCR1*), opsonization (*FCN1*), and phagocytosis (CD36). For a detailed probe set listing, see Table V.

Comparison of 'Treated vs. Healthy' group. In this comparative group, in total, 43 differentially expressed genes could be detected. As seen in Table VI, 39 probe sets were relatively down-regulated in 'Treated' patients. Amongst others, gene products that could be attributed to immune response such as *CCR7* and *IL-7* receptors or to chemotaxis

Table V. *Differentially expressed genes in "Non-treated vs. Healthy" group of comparison. Change Call: Increase.*

Probe set ID	Gene symbol	Gene title	GO biological process term	Cytoband location	Mean fold change
211307_s_at	*FCAR*	Fc fragment of IgA, receptor for	Immune response	19q13.2-q13.4	24,25
216950_s_at	*FCGR1A*	Fc fragment of IgG, high affinity Ia, receptor (CD64)	Phagocytosis,	1q21.2-q21.3	18,66
	FCGR1C	Fc fragment of IgG, high affinity Ic, receptor (CD64)	Immune reponse		
214511_x_at	*FCGR1B*	Fc fragment of IgG, high affinity Ib, receptor (CD64)	Immune response	1p11.2	16,55
205568_at	*AQP9*	aquaporin 9	Immune response	15q	10,79
235568_at	*C19orf59*	Chromosome 19 open reading frame 59	-	19p13.2	9,50
210119_at	*KCNJ15*	Potassium inwardly-rectifying channel, subfamily J, member 15	Potassium ion transport	21q22.2	9,20
206111_at	*RNASE2*	Ribonuclease, RNase A family- 2 (liver, eosinophil-derived neurotoxin)	Chemotaxis	14q24-q31	8,67
202912_at	*ADM*	Adrenomedullin	Cell-cell signaling	11p15.4	8,54
202241_at	*TRIB1*	Tribbles homolog 1 (Drosophila)	Negative regulation of protein kinase activity	8q24.13	7,31
218454_at	*PLBD1*	phospholipase B domain containing 1	Lipid catabolic process	12p13.1	6,98
205119_s_at	*FPR1*	Formyl peptide receptor-1	Chemotaxis	19q13.4	6,97
225987_at	*STEAP4*	STEAP family member 4	Ion transport	7q21.12	6,94
206488_s_at	*CD36*	CD36 molecule (thrombospondin receptor)	Positive regulation of phagocytosis, positive regulation of IL-6, IL-12 and TNF production	7q11.2	6,81
219434_at	*TREM1*	Triggering receptor expressed on myeloid cells 1	Humoral immune response	6p21.1	6,80
211571_s_at	*VCAN*	Versican	Cell adhesion	5q14.3	6,07
209616_s_at	*CES1*	Carboxylesterase 1	Response to toxin	16q22.2	6,03
204614_at	*SERPINB2*	Serpin peptidase inhibitor, clade B (ovalbumin), member 2	Anti-apoptosis, wound healing	18q21.3	5,98
207677_s_at	*NCF4*	Neutrophil cytosolic factor 4, 40kDa	Immune response	22q13.1	5,85
222670_s_at	*MAFB*	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)	Regulation of transcription	20q11.2-q13.1	5,34
221210_s_at	*NPL*	N-acetylneuraminate pyruvate lyase (dihydrodipicolinate synthase)	Metabolic process	1q25	5,16
203922_s_at	*CYBB*	Cytochrome b-245, beta polypeptide	Innate immune response	Xp21.1	5,06
206522_at	*MGAM*	Maltase-glucoamylase (alpha-glucosidase)	Metabolic process	7q34	5,03
206643_at	*HAL*	Histidine ammonia-lyase	Histidin metabolic process	12q22-q24.1	4,97
203821_at	*HBEGF*	Heparin-binding EGF-like growth factor	Positive regulation of cell migration	5q23	4,66
205237_at	*FCN1*	Ficolin (collagen/fibrinogen domain containing)	Opsonization	9q34	4,18
205098_at	*CCR1*	Chemokine (C-C motif) receptor 1	Chemotaxis, immune response	3p21	4,16
207111_at	*EMR1*	egf-like module containing, mucin-like, hormone receptor-like 1	Cell adhesion, signal transduction	19p13.3	4,05
223922_x_at	*MS4A6A*	Membrane-spanning 4-domains, subfamily A, member 6A	-	11q12.1	4,03
224917_at	*MIR21*	microRNA-21	-	17q23.1	3,79
208130_s_at	*TBXAS1*	Thromboxane A synthase 1 (platelet)	Prostaglandin biosynthesic process	7q34-q35	3,79
220615_s_at	*FAR2*	Fatty acyl CoA reductase 2	Lipid metabolic process	12p11.22	3,70
208892_s_at	*DUSP6*	Dual specificity phosphatase 6	Response to growth factor stimulus	12q22-q23	3,53
227697_at	*SOCS3*	Suppressor of cytokine signaling 3	Response to cytokine stimulus	17q25.3	3,37
213418_at	*HSPA6*	Heat shock 70kDa protein 6 (HSP70B')	Response to stress	1q23	2,50

such as *CXCR3* and *LEF1*, were relatively overexpressed up to nine-fold in the group 'Healthy'. Compared to 'Healthy' subjects, 'Treated' HNSCC patients showed a relative up-regulation of four genes that, amongst others, could be attributed to biological processes such as cell chemotaxis (*PDGFRB*) or excretion (*DAB2*) (Table VII).

Differentially expressed genes in more than one comparative group. In order to detect more candidate genes, we expanded the inclusion criteria detection call 'present' from 15 to 13 of 16 cross-comparisons. The mean fold change value remained ≥2.0. Here, 15 relatively up-regulated genes could be detected in the group 'Non-treated' when

Table VI. *Differentially expressed genes in the Treated vs. Healthy' group of comparison. Change Call: Decrease.*

Probe Set ID	Gene symbol	Gene title	GO biological process term	Cytoband location	Mean fold change
241871_at	*CAMK4*	Calcium/calmodulin-dependent protein kinase IV	Signal transduction	5q21.3	–22,74
212730_at	*SYNM*	Synemin, intermediate filament protein	Cytosceleton organization	15q26.3	–11,88
210439_at	*ICOS*	inducible T-cell co-stimulator	Immune response	2q33	–11,16
215332_s_at	*CD8B*	CD8b molecule	Immune response	2p12	–10,85
244261_at	*IL28RA*	Interleukin-28 receptor, alpha (interferon, lambda receptor)	Regulation of defense response to virus by host	1p36.11	–9,52
230489_at	*CD5*	CD5 molecule	Induction of apoptosis by extracellular signals, T-cell co-stimulation	11q13	–9,31
206337_at	*CCR7*	Chemokine (C-C motif) receptor 7	Chemotaxis, immune response	17q12-q21.2	–8,96
217147_s_at	*TRAT1*	T-cell receptor associated transmembrane adaptor-1	Cellular defense response	3q13	–8,25
213539_at	*CD3D*	CD3d molecule, delta (CD3-TCR complex)	Positive thymic T cell selection	11q23	–7,67
206181_at	*SLAMF1*	Signaling lymphocytic activation molecule family member-1	Lymphocyte activation	1q23.3	–7,47
226272_at	*RCAN3*	RCAN family member-3	Calcium-mediated signaling	1p35.3-p33	–7,13
39248_at	*AQP3*	Aquaporin-3 (Gill blood group)	Positive regulation of immune system	9p13	–7,06
213534_s_at	*PASK*	PAS domain containing serine/threonine kinase	Regulation of transcription	2q37.3	–6,95
1556839_s_at	Hypothetical protein LOC100289090	LOC100289090	–	15q15.1	–6,84
206804_at	*CD3G*	CD3g molecule, gamma (CD3-TCR complex)	T-cell activation	11q23	–6,45
227867_at	Chromosome 2 open reading frame 89	C2orf89	–	2p11.2	–6,44
203413_at	*NELL2*	NEL-like 2 (chicken)	Cell adhesion	12q12	–6,02
210972_x_at	*RAC*	T-cell receptor alpha constant	Cellular defense response	14q11	–5,85
	TRAJ17	T-cell receptor alpha joining 17			
	TRAV20	T-cell receptor alpha variable 20			
205798_at	*IL7R*	Interleukin-7 receptor	Immune response	5p13	–5,70
209670_at	*TRAC*	T-cell receptor alpha constant	Cellular defense response	14q11	–5,56
230005_at	*SVIP*	Small VCP/p97-interacting protein	–	11p14.2	–5,44
211207_s_at	*ACSL6*	Acyl-CoA synthetase long-chain family member-6	Positive regulation of plasma membrane long-chain fatty acid transport	5q31	–5,39
205376_at	*INPP4B*	Inositol polyphosphate-4-phosphatase, type II, 105kDa	Signal transduction	4q31.21	–4,92
1555579_s_at	*PTPRM*	Protein tyrosine phosphatase, receptor type, M	Cell adhesion	18p11.2	–4,32
230110_at	*MCOLN2*	Mucolipin 2	Ion transport	1p22	–4,15
207681_at	*CXCR3*	Chemokine (C-X-C motif) receptor 3	Chemotaxis	Xq13	–4,11
221558_s_at	*LEF1*	Lymphoid enhancer-binding factor 1	Chemotaxis, cell migration, negative regulation of IL-4, 5 and 13 production	4q23-q25	–4,05
206150_at	*CD27*	CD27 molecule	Immunoglobulin-mediated immune response, induction of apoptosis	12p13	–3,96
203130_s_at	*KIF5C*	Kinesin family member 5C	Organelle organization	2q23.1	–3,93
206914_at	*CRTAM*	Cytotoxic and regulatory T-cell molecule	positive regulation of natural killer cell mediated cytotoxicity directed against tumor cell target	11q24.1	–3,72
202431_s_at	*MYC*	v-myc myelocytomatosis viral oncogene homolog (avian)	Regulation of transcription, activation of pro-apoptotic gene products	8q24.21	–3,60
207339_s_at	*LTB*	Lymphotoxin beta (TNF superfamily, member 3)	Immune response, positive regulation of IL-12 biosynthetic process	6p21.3	–3,42
205259_at	*NR3C2*	Nuclear receptor subfamily 3, group C, member 2	Regulation of transcription	4q31.1	–3,41
222557_at	*STMN3*	stathmin-like 3	Regulation of cytoskeleton organization	20q13.3	–3,09
210607_at	*FLT3LG*	fms-related tyrosine kinase 3 ligand	Positive regullation of cell proliferation	19q13.3	–2,69
231775_at	*TNFRSF10A*	Tumor necrosis factor receptor superfamily, member 10a	Induction of apoptosis *via* death domain receptors (TRAIL)	8p21	–2,56
206980_s_at	*FLT3LG*	fms-related tyrosine kinase 3 ligand	Positive regulation of cell proliferation	19q13.3	–2,48
205758_at	*CD8A*	CD8a molecule	Immune response	2p12	–2,26
205255_x_at	*TCF7*	Transcription factor 7 (T-cell specific, HMG-box)	Regulation of transcription, immune response	5q31.1	–2,21

Table VII. *Differentially expressed genes in the 'Treated vs. Healthy' group comparison. Change Call: Increase.*

Probe set ID	Gene symbol	Gene title	GO biological process term	Cytoband location	Mean fold change
201279_s_at	*DAB2*	Disabled homolog 2, mitogen-responsive phosphoprotein (Drosophila)	Excretion, regulation of transcription	5p13	3,41
232720_at	*LINGO2*	Leucine rich repeat and Ig domain containing-2	-	9p21.2	3,27
202273_at	*PDGFRB*	Platelet-derived growth factor receptor, beta polypeptide...	Cell chemotaxis	5q33.1	2,78
201162_at	*IGFBP7*	Insulin-like growth factor binding protein-7	Negative regulation of cell proliferation, cell adhesion	4q12	2,42

comparing the expression levels with 'Treated' patients or 'Healthy' subjects. Likewise, 'Healthy' individuals showed a relative overexpression of 13 genes compared to the groups 'Non-treated' and 'Treated'.

The detailed listing of candidate genes is shown in Table VIII.

Discussion

Increasing insights into the function of the immune system provide novel approaches in understanding tumor biology. Impaired immune surveillance is one probable cause of cancer genesis and its progression. The development of HNSCC is highly influenced by the host immune system, in which T-cells continue to be considered the critical immune cells involved in antitumor immunity (5, 6). NK cells are known to be the central component of the innate immune system capable of eliminating malignantly transformed cells (15). Several studies have shown immune cell dysfunction in HNSCC patients as a consequence of tumor manifestation (6, 11, 22). This circumstance may be, in part, explained by tumor immune escape mechanisms, in which 'tumor counter-attacks' such as FasL-expression, TRAIL, and TNF-α pathways play a distinct role (23, 24). HNSCC cell lines are also able to produce cytokines such as TGF-β1 and IFN-γ or to overexpress matrix metalloproteinases and ADAMs (a disintegrin and metalloproteinases), which inhibit NK cell function (25, 26).

Our previous results have shown significantly increased proportions of NK cells in PBMC in treated, relapse-free HNSCC patients compared to healthy subjects or non-treated patients (20, 27). In the present study, we demonstrated changes in expression levels of different genes in NK cells, which may explain several clinical circumstances in patients with HNSCC.

Possible reasons for changes in distribution. Concerning increased proportions of NK cells in PBMC of treated, relapse-free HNSCC patients, we could demonstrate that a 4.84-fold down-regulation of the suppressor of cytokine signaling-3 (*SOCS3*) was present in 'Treated' compared to 'Non-treated' subjects. *SOCS3* is known to be a regulator of IL-6 and IL-10 pathways and plays a decisive role in lymphocyte development (28). Thus, a negative regulation of NK cell development could be assumed.

Expression levels of cyclin-dependent kinase-5, regulatory subunit-1 (*p35*) (*CDK5R1*) appeared to be 2.66-fold higher in 'Treated' *vs.* 'Non-treated' patients and is attributed to TNF-α-dependent signaling (29) and cell migration (30), which may lead to higher peripheral NK cell counts.

Delta-like-1 (Drosophila) (*DLL1*), a human homolog of the Notch Delta ligand interacting with Notch-2, was 4.29-fold up-regulated in 'Treated' patients compared to 'Non-treated'. This is attributed to regulation of cell fate during hematopoiesis. Blocking of the notch signaling pathway leads to increased appearance of NK cell progenitors (31, 32).

Another candidate gene for clarifying changes in the distribution of NK cells is the platelet-derived growth factor receptor, beta polypeptide (*PDGFRB*) which was 2.78-times overexpressed in 'Treated' subjects compared to 'Healthy' ones. It promotes autocrine survival and is up-regulated in large granular lymphocyte leukemia (33).

Insulin-like growth factor binding protein-7 (*IGFBP7*) is associated with leukemia. It is co-expressed with CD34 in regular progenitors of hematopoiesis (34) and it regulates proliferation of leukemic cells (35). In NK cells of relapse-free, treated patients, a 2.42-fold up-regulation of *IGFBP7* became evident compared to 'Healthy' probands.

Arguments for impaired immune surveillance. Focusing on impaired immune surveillance as a reason for manifestation of a malignant tumor, we could demonstrate that, amongst others, expression levels of cytokine receptors such as *CCR7, IL-7R,* and *CXCR3* were down-regulated up to 11.97-fold in 'Non-treated' HNSCC patients compared to 'Healthy' subjects.

Table VIII. *Differentially expressed genes in more than one group comparison Change Call: Increase and Decrease. Grey fields=no change.*

Gene symbol	Gene title; (GO biological process term)	Treated *vs*. Non-treated Mean fold change	Treated *vs*. Healthy Mean fold change	Non-treated *vs*. Healthy Mean fold change
F5	Coagulation factor V (proaccelerin, labile factor); (oxidation reduction)	–7,29		10,86
C19orf59	Chromosome 19 open reading frame 59; (–)	–8,49		9,50
KCNJ15	Potassium inwardly-rectifying channel, subfamily J, member 15; (potassium ion transport)	–21,51		9,20
CLU	Clusterin; (response to stress, complement activation)	–12,93		8,57
ADM	Adrenomedullin; (Cell-cell signaling, response to wounding)	–7,62		8,54
CD36	CD36 molecule (thrombospondin receptor); (positive regulation of IL-12, 6, and TNF production)	–8,70		8,42
SLC11A1	Solute carrier family-11 (proton-coupled divalent metal ion transporters), member 1; (immune response, IFN-γ, IL-2 and 3 production)	–4,91		7,88
PLBD1	Phospholipase B domain containing-1; (Lipid catabolic process)	–6,64		6,98
CES1	carboxylesterase 1; (Response to toxin)	–5,09		6,03
CLEC4D	C-type lectin domain family 4, member D; (innate immune response)	–6,35		6,01
MAFB	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian); (regulation of transcription)	–6,29		5,34
HBEGF	Heparin-binding EGF-like growth factor; (Positive regulation of cell migration)	–4,89		4,66
CCR1	chemokine (C-C motif) receptor 1; (Chemotaxis, immune response)	–3,94		4,16
MIRN21	microRNA 21; (–)	–4,86		3,79
SOCS3	Suppressor of cytokine signaling-3; (Response to cytokine stimulus)	–4,84		3,37
CXCR3	Chemokine (C-X-C motif) receptor-3; (chemotaxis)		–4,11	–5,28
LOC100289090	Hypothetical protein LOC100289090; (–)		–6,84	–5,44
TRAT1	T-cell receptor associated transmembrane adaptor 1; (cellular defense response)		–8,25	–5,95
CD5	CD5 molecule; (Induction of apoptosis by extracellular signals)		–9,31	–8,36
PASK	PAS domain containing serine/threonine kinase; (regulation of transcription)		–6,95	–8,64
NELL2	NEL-like 2 (chicken); (cell adhesion)		–6,02	–10,28
CD8B	CD8b molecule; (immune response)		–10,85	–10,61
IL7R	Interleukin 7 receptor; (immune response)		–6,91	–10,70
CCR7	Chemokine (C-C motif) receptor-7; (chemotaxis, immune response)		–8,96	–11,97
CD8B	CD8b molecule (immune response)		–8,55	–12,87
SYNM	Synemin, intermediate filament protein; (cytoskleleton organization)		–11,88	–20,97
NOG	Noggin; (negative regulation of cytokine activity)		–49,37	–52,51
TRD@	T-cell receptor delta locus; (cellular defense response)		–14,36	–85,34

Interleukin-7 receptor α (*IL-7R*) is required for normal lymphoid development. Defective expression of this receptor causes severe immune deficiency (36, 37). *CXCR3*, the receptor for the chemokines *CXCL9, CXCL10*, and *CXCL11*, is responsible for chemotaxis. Deficiency of *CXCR3* or its ligands significantly impairs cell-mediated immunity (38, 39).

Sphingosine-1-phosphate receptor (*S1PR1*) is attributed to positive regulation of chemotaxis and is relatively under-expressed in non-treated patients (40). Down-regulation of *S1PR1* could be a reason for impaired *PDGF*-induced NK cell migration in cancer patients.

Though unusual for NK cells, surface molecules *CD5* and *CD8b* expressed in certain subsets (41, 42) are relatively down-regulated in HNSCC patients with no prior therapy compared to healthy individuals. *CD5*, attributed to induction of apoptosis and regulation of cell differentiation, and *CD8b*, acting as a co-receptor in cell–cell interaction in immune response, are under-expressed up to 8.36-fold and 12.87-fold, respectively, in non-treated patients indicating a probable cause for impaired immune surveillance.

Possible responses to malignancy manifestation. Compared to healthy subjects, NK cells of non-treated HNSCC patients show a variety of differentially expressed genes that could be interpreted as an immunological response to tumor manifestation. The chemokine receptor *CCR7*, attributed to migration of NK cells to lymph nodes (43), is down-regulated by 11.97-fold in 'non-treated' patients. This may be explained by the need for peripheral circulating killers as a response to malignancy.

In NK cells of non-treated HNSCC patients, several important genes that are associated with immune response were up-regulated up to 24.25-fold compared to healthy subjects (*e.g.* receptors for Fc-fragments of specific antibodies or aquaporin 9) as seen in Table V. One could assume an increased ADCC after tumor manifestation. Besides those, other genes responsible for chemotaxis (*FPR1* (44) and *CCR1* (45)), phagocytosis (*CD36* (46)), and opsonization, such as the phagocytosis receptor ficolin-1 (*FCN1*) (47), are up-regulated in NK cells of cancer patients compared to healthy individuals, which indicates an increased immune response in the presence of HNSCC.

A relative 3.98-fold underexpression of the p53 apoptosis effector related to PMP-22 (*PERP*) in 'Non-treated' patients became evident in comparison to healthy subjects. *PERP* is a direct p53-target and induces cell death in various cell types. *PERP*-deficiency enhances cell survival and inflammation in a murine skin model (48, 49). Many other candidate genes that could account for a higher anti-tumorous activity in NK cells after HNSCC manifestation are included in Table V.

Conclusion

Our study provides novel insight into gene expression changes in NK cells of HNSCC depending on patient's clinical status. We found several up-regulated candidate genes in recurrence-free patients that seem to cause distribution changes such as increased proportions of circulating NK cells. In addition, we detected certain overexpressed genes in NK cells that could be interpreted as an increased anti-tumor response to malignancy manifestation.

Most notably, in NK cells we discovered several down-regulated genes, indicating an impaired immune surveillance as a most probable reason for tumorigenesis leading to HNSCC.

Conflicts of Interest

R. Knecht is a member of the Advisory Board of Merck Serono, Sanofi Aventis, Boehringer Ingelheim and Bayer Healthcare, Leverkusen. The other Authors report no conflicts of interest.

Acknowledgements

We would like to thank Ms. Ildiko Todt from the Institute of Immunology, University of Rostock for helpful preparatory work in the laboratory.

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Received August 8, 2013
Revised September 16, 2013
Accepted September 16, 2013