Analysis of chemokine and chemokine receptor expression in squamous cell carcinoma of the head and neck (SCCHN) cell lines

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Abstract The purpose of this work was to analyze chemokine and chemokine receptor expression in untreated and in irradiated squamous cell carcinoma of the head and neck (SCCHN) tumor cell lines, aiming at the establishment of assays to test for the relevance of chemokine and chemokine receptor expression in the response of SCCHN to radiotherapy and radiochemotherapy. Five low passage and 10 established SCCHN lines, as well as two normal cell lines, were irradiated at 2 Gy or sham-irradiated, and harvested between 1 and 48 h after treatment. For chemokines with CC and CXC structural motifs and their receptors, transcript levels of target and reference genes were quantified relatively by real-time PCR. In addition, CXCL1 and CXCL12 protein expression was analyzed by ELISA. A substantial variation in chemokine and chemokine receptor expression between SCCHN was detected. Practically, all cell lines expressed CCL5 and CCL20, while CCL2 was expressed in normal cells and in some of the tumor cell lines. CXCL1, CXCL2, CXCL3, CXCL10, and CXCL11 were expressed in the vast majority of the cell lines, while the expression of CXCL9 and CXCL12 was restricted to fibroblasts and few tumor cell lines. None of the analyzed cell lines expressed the chemokines CCL3, CCL4, or CCL19. Of the receptors, transcript expression of CCR1, CCR2, CCR3, CCR5, CCR7, CCXR2, and CCXR3 was not detected, and CCR6, CXCR1, and CXCR4 expression was restricted to few tumor cells. Radiation caused up- and down-regulation with respect to chemokine expressions, while for chemokine receptor expressions down-regulations were prevailing. CXCL1 and CXCL12 protein expression corresponded well with the mRNA expression. We conclude that the substantial variation in chemokine and chemokine receptor expression between SCCHN offer opportunities for the establishment of assays to test for the relevance of chemokine and chemokine receptor expression in the response of SCCHN to radiotherapy and radiochemotherapy.

Introduction

Tumors are embedded in a rich cell microenvironment, which is essential for tumor cell survival, cancer progression, and metastasis. However, the means by which tumor cells interact with their surrounding are mostly unknown. Chemokine molecules constitute a superfamily of often inducible, secreted, proinflammatory proteins involved in a variety of immune responses, acting primarily as chemotaxants and activators of specific types of leukocytes.
They are typically induced by inflammatory cytokines, growth factors, and pathogenic stimuli and signal through transmembrane G-protein-coupled (chemokine) receptors. Chemokines are made and secreted by many different cell types, including tumor cells, tumor stroma cells, and tumor-infiltrating immune cells (Zlotnik 2006). Recent studies have shown the involvement of chemokine signaling in cancer treatment response and metastasis through autocrine and paracrine mechanisms (Wang et al. 2009; Zlotnik 2006).

Radiotherapy is an established treatment option for numerous tumors, and evidence is accumulating that radiation has considerable effects on chemokine expression (Ao et al. 2009; Facoetti et al. 2009; Gremy et al. 2008; Johnston et al. 2002; im et al. 2009; Kuhlmann et al. 2009; Linard et al. 2004; Lugade et al. 2008; Matsumura et al. 2008; Mihaescu et al. 2010; Moriconi et al. 2008; Muller and Meineke 2007; Sanzari et al. 2009; Schmidtner et al. 2009), while radiation effects on chemokine receptor expression are poorly investigated (Johnston et al. 2002; Malik et al. 2010). Experimental studies analyzed normal cells (Facoetti et al. 2009; Kuhlmann et al. 2009; Moriconi et al. 2008; Muller and Meineke 2007) or tissues (Ao et al. 2009; Gremy et al. 2008; Johnston et al. 2002; Linard et al. 2004; Malik et al. 2010; Mihaescu et al. 2010; Moriconi et al. 2008), as well as tumor cells of different origin (Kim et al. 2009; Lugade et al. 2008; Matsumura et al. 2008; Sanzari et al. 2009; Schmidtner et al. 2009).

Squamous cell carcinoma of the head and neck (SCCHN) has been shown to express a number of chemokines, and their receptors, which may e.g., promote chemotherapy resistance (Muller et al. 2006; Wang et al. 2008) or may allow them to access the lymphatic system and spread to regional lymph nodes (Samara et al. 2004; Ueda et al. 2009; Wang et al. 2004, 2005a, b). Schmidtner et al. reported on radiation-associated escalation of the chemokine CCL22 in SCCHN tumor cell supernatants, which might modify the transmigration of tumor-infiltrating lymphocytes beneficially and thereby support the immune response (Schmidtner et al. 2009).

We are aiming at the establishment of assays to test for the relevance of chemokine and chemokine receptor expression in the response of SCCHN to radiotherapy and radiochemotherapy. While searching the literature, we found that there are practically no published data on chemokine and chemokine receptor expression in commercially available SCCHN tumor cell lines. Therefore, we analyzed the transcript expression of 24 genes (Table 1), involving chemokines and chemokine receptors in 15 SCCHN and 2 normal cell lines; for validation, extracts pooled from cells with known or putative chemokine and chemokine receptor expression were analyzed as well.

The analyzed genes were selected with respect to our experiences concerning radiation-dependent cytokine/chemokine regulation (Malik et al. 2010; Moriconi et al. 2008) and with respect to published reports on chemokine expression in head and neck cancer (Abiko et al. 2003; Almofti et al. 2004; Chakraborty et al. 2008; Chang et al. 2008; Delibasli et al. 2004; Ferreira et al. 2008; Ishikawa et al. 2006; Khademi et al. 2008; Michiels et al. 2009; Miyazaki et al. 2006; Muller et al. 2006; Oliveira-Neto et al. 2008; Rehman and Wang 2008; Takes et al. 2008; Tan et al. 2008; Tsuzuki et al. 2006; Ueda et al. 2009; Wang et al. 2005, 2008; Wen et al. 2008; Yeudall and Miyazaki 2007).

Materials and methods

The SCCHN cell lines used were either established from tumor material and used at low passage numbers (Rave-Frank et al. 1996) or commercially available (established cell lines)

Table 1 Chemokines and chemokine receptors of which the transcript expression was analyzed

| Chemokine | Other names | Full name | Receptor |
|-----------|-------------|-----------|----------|
| CCL2      | MCP-1, SCYA-2 | Monocyte chemoattractant protein 1 | CCR2 |
| CCL3      | MIP-1α, SCYA3 | Macrophage inflammatory protein 1 alpha | CCR1, 5 |
| CCL4      | MIP-1β, SCYA4 | Macrophage inflammatory protein 1 beta | CCR5, 8 |
| CCL5      | RANTES | Regulated upon activation of normal T cell | CCR1, 3, 5 |
| CCL19     | MIP3-α, SCYA19 | Macrophage inflammatory protein 3 beta | CCR7 |
| CCL20     | MIP3-β, LARC | Macrophage inflammatory protein 3 alpha | CCR6 |
| CXCL1     | GROα, SCYB1 | Growth-related oncogene alpha | CXCR1, 2 |
| CXCL2     | GROβ, SCYB2 | Growth-related oncogene beta | CXCR1, 2 |
| CXCL3     | GROγ, SCYB3 | Growth-related oncogene gamma | CXCR1, 2 |
| CXCL9     | MIG, SCYB9 | Monokine induced by interferon gamma | CXCR3 |
| CXCL10    | IP-10, SCYB10 | Interferon-inducible protein-10 kilodaltons | CXCR3 |
| CXCL11    | I-TAC, SCYB9B | Interferon-inducible T-cell alpha chemoattractant | CXCR3 |
| CXCL12    | SDF-1, SCYB12 | Stromal cell-derived factor 1 | CXCR4 |
(Table 2). For experiments, cells were grown in their recommended media (Table 2), irradiated at 2 Gy or sham-irradiated, and harvested 6, 24, or 48 h upon treatment. Cell pellets and cell supernatants were stored at $-80^\circ$C. For real-time PCR, cells were washed in PBS and stored at $-80^\circ$C in lysis buffer contained in RNeasy mini kit (Qiagen, Hilden, Germany). Total cell amount per sample was about 1 million cells. RNA was isolated using Rneasy mini kit according to the manufacturer’s instructions. The amount of RNA was meticulously quantified by a spectrophotometric method specifically designed for precise measurements of undiluted samples in low microliter scale (LabelGuard cuvette; Implen, Munich, Germany). Reverse transcription to complementary DNA was effected for 1 h with 1 $\mu$g total RNA using Super-Script II reverse transcriptase (Invitrogen, Carlsbad, California, USA) and 20 units per sample recombinant RNase inhibitor (USB, Cleveland, Ohio, USA). Transcript numbers of target and reference genes were quantified relatively by real-time PCR using HotStart-IT SYBR Green qPCR-Master Mix (USB, Cleveland, Ohio, USA) in a real-time PCR machine (HT7900, Applied Biosystems, Foster City, California, USA). PCR conditions were 40–50 cycles (dependent on expression level) with annealing at 60°C for 20 s and elongation at 72°C for 40 s for each primer pair. Specific primer pairs were synthesized by MWG, Ebersberg, Germany. Efficacy and specificity of primers were tested by serial dilution (factor 625). Primer sequences are given in Table 3. Data were normalized to weighted mean expression of HPRT1 and UBC serving as reference genes. The analysis was performed for cells harvested 6 h after treatment and sham-treated cells, respectively. Greater than twofold changes in transcript expression were taken as significant.

The amount of CXCL1 or CXCL12 protein was determined in cell culture supernatants by the Quantikine® Human CXCL1 or CXCL12 Immunoassays, R&D Systems, Minneapolis, USA, according to recommended protocols. For experiments, tumor cells, HaCat-keratinocytes, or fibroblasts (1,000,000 cells/flask) were seeded in 25-cm² flasks (Nalge Nunc Int., Rochester, NY) in their specific medium and allowed to attach. Measurements were repeated 3 times, and the statistical significance of increased chemokine concentrations was tested by ANOVA.
Table 3  Primer pairs used for the analysis of chemokine and chemokine receptor transcript expressions

| Gen   | Forward primer (5′→3′)        | Reverse primer (5′→3′)        | AL (bp) | Source                                      |
|-------|-------------------------------|-------------------------------|---------|---------------------------------------------|
| CCL2  | CAGCCAGATGAATCATATGCC         | TGGAAATCCGTGAACCCACTCTTCT     | 190     | PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html) |
| CCL3  | AGTCTCTCTGCTACACTTGTG         | CGGCTTGCTGGTGTAGAGGA          | 151     | PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html) |
| CCL4  | TCTGTCGTGATCCCCAGTGAAAT       | TCAGTTCACCTTACCACTACA         | 61      | PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html) |
| CCL5  | ATCTCCTATTGCTACTGCCTCT        | GCCACTTGTTGAAAATACCTC         | 135     | PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html) |
| CCL19 | CCAAGCCCCACTCTGAGTG           | ATCTCTATGAGAAGGTAGTTGA        | 114     | PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html) |
| CCL20 | TGCTGTAACAAAGGTGGTGTCTC       | GCACACAGAACAATTTTTTCTT       | 220     | PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html) |
| CXCL1 | CTTGCACTCATCCTGCATC           | CTTCTCCTGAGTTGAGATTG         | 82      | Bachmeier et al. (2008)                     |
| CXCL2 | CGAAGTCATAGCGGACACTCAAGG      | CTCTCTGTCATGGGATTGG           | 116     | Bachmeier et al. (2008)                     |
| CXCL3 | GCAAGGAAATTCTACCTCAAGA        | GGTGCTTCCCTTGTCCAGTA          | 172     | Kato et al. (2009)                          |
| CXCL9 | TGCAAGGAACCCAGTAAGTG          | GGTGAGATGTCCTTGTTGGG          | 69      | Okamoto et al. (2008)                       |
| CXCL10| GAGCTCAGACAGCAGGAACC          | GAGTCAGAAAGATAAGGCAGG         | 81      | Okamoto et al. (2008)                       |
| CXCL11| GCTGGTGAATTGGTGCTACTAGT       | GCTTGATTTTGGGATTGCA           | 211     | PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html) |
| CXCL12| GGTCTGCTGTGGTGTGCTGT          | GGGCCTAATCTGAGGGG             | 174     | Zhou et al. (2008)                          |
| CCR1  | CACGCCAAGACGCCCTCAGGG         | TGTGCTTGTGCTCAGTGCTCTG        | 79      | Deutsch et al. (2008)                       |
| CCR2  | GCCCTTTCACTAGCTCTGGGC         | AGAGATCCTTGTGAGTCCTT          | 112     | PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html) |
| CCR3  | ATACAGGAAGGTGGCAGATAGATG      | ATGCCCCCTGACATAGTGAT          | 109     | PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html) |
| CCR5  | ACCAGCTATGCCCAGTGAGCAG        | GCAGAAGCGTTGGCAGAGG           | 153     | PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html) |
| CCR6  | CTCAAGGCTATTTTGTACCAGATAGT    | CACTGCCAGAATGGGAGG            | 181     | PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html) |
| CCR7  | GGGCAGACCGCTCTCCTG           | CCACCAGACGCACGCTT            | 82      | Deutsch et al. (2008)                       |
| CCR8  | TGCCCTCTGTTGATTCACTGCT        | CACTTTTGACATTACAGTCTCCA       | 198     | PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html) |
| CCR1  | GCAGCTCTCTCACTGTGGGACACA      | CATGTGCTCTCATGGTCAGCACA       | 75      | Deutsch et al. (2008)                       |
| CCR2  | GCAGCTACAGCTGCTTCCTCT         | TGGAGTAAACACTTAATCCTGACTG     | 82      | Deutsch et al. (2008)                       |
| CCR3  | ACCAGGCGACCGAGGACCA          | CATAGAGAAGCTGAGTGTCCAGAG     | 109     | Deutsch et al. (2008)                       |
| CCR4  | TACACCAGGAAGAATGGGCTCA        | AGATGATGAGATAGCTGAGGG         | 112     | PrimerBank (http://pga.mgh.harvard.edu/primerbank/index.html) |
| HPRT1 | TGACACTGGCAAAAACAAATGCA       | GGTCTTTTTCACAGAACAGCT        | 93      | Calcagni et al. (2006)                      |
| UBC   | CGGTGAAACCGCGATGATTAT         | ATCTGCATTGCTAAGTGACA         | 123     | Cicinnati et al. (2008)                     |
Results

Within the pooled validation samples consisting of extracts from human lymphocytes, colorectal, and hepatocellular cancer cells, significant transcript expression of all analyzed chemokines and chemokine receptors was detected; the respective CT-values are given in Table 4.

Normal cells, as represented by dermal fibroblasts and HaCat-keratinocytes, showed comparable expression patterns, namely the expression of a variety of chemokines and no chemokine receptors. The transcript expression data for normal cells are integrated in Figs. 1, 2, 3, 4.

Practically, all cell lines expressed the CC-chemokines CCL5 and CCL20, while CCL2 was expressed in normal cells and in some of the tumor cell lines (BHY, CAL27, CAL33, HSC4, SAS, BW-225, ZMK-1) (Fig. 1a–c). Likewise, the CXC-chemokines CXCL1, CXCL2, CXCL3, CXCL10, and CXCL11 were expressed in the vast majority of the cell lines, while the expression of CXCL9 and CXCL12 was restricted to fibroblasts and the tumor cell lines CAL27, CAL33, XF354, BW-225, and GR-145-I/II, HK-147, BW-225, respectively. Data representing transcript expressions of CXCL1, 2, 3 are given in Fig. 2 a–c; data representing transcript expressions of CXCL10, 11, 9, 12 are given in Fig. 3 a–d. None of the analyzed cell lines expressed the chemokines CCL3, CCL4, and CCL19.

Of the receptors, transcript expression of CCR1, CCR2, CCR3, CCR5, CCR7, CCR2, CCXR3 was not detected, and CCR6 expression was restricted to BHY, KOSC2, SKN3, GR-145-I, and HK-147 tumor cells (Fig. 4a). The CXC-chemokine receptors were expressed in different cell lines; CXCR1 was expressed in HaCat, CAL33, HSC4, and XF354 (Fig. 4b), and CXCR4 was expressed in HO 1u1, FaDu, BW-225, and ZMK-1 (Fig. 4c). Radiation caused up- and down-regulation with respect to chemokine expressions, while for chemokine receptor expressions down-regulations were prevailing. Greater than twofold radiation-related changes were regarded as significant and are marked by asterisks in the respective figures.

To test whether mRNA transcript expression was translated into protein, the presence of CXCL1 and CXCL12 was determined in cell culture supernatants by ELISA. Figure 5a, b show that the protein expression matches the mRNA transcript data well; however, no significant differences between irradiated and sham-irradiated cells were detected. Furthermore, Fig. 6 demonstrates that CXCL12 accumulates in irradiated cell culture supernatants over time, the increase being significant for tumor (BW-225) and normal (DF-19) cells.

Table 4 Transcript expression of chemokines and chemokine receptors in the pooled sample

| Chemokine | C_T  | Chemokine receptor | C_T  |
|-----------|------|--------------------|------|
| CCL2      | 20.33| CCR1               | 30.80|
| CCL3      | 26.08| CCR2               | 29.07|
| CCL4      | 29.67| CCR3               | 28.58|
| CCL5      | 26.43| CCR5               | 29.44|
| CCL19     | 30.15| CCR6               | 22.77|
| CCL20     | 18.07| CCR7               | 30.12|
| CXCL1     | 19.06| CCR8               | 29.44|
| CXCL2     | 19.89| CCR1               | 29.85|
| CXCL3     | 20.58| CCR2               | 31.79|
| CXCL9     | 29.66| CCR3               | 27.18|
| CXCL10    | 26.91| CCR4               | 24.84|
| CXCL11    | 25.99|                    |      |
| CXCL12    | 30.03|                    |      |

Fig. 1 CC-chemokine transcript expression relative to housekeeping genes in tumor and normal cell lines; a CCL5, b CCL20, c CCL2. Greater than twofold expression changes were regarded as significant and are marked by asterisks.
Discussion

Aim of the present study was the analysis of chemokine and chemokine receptor expression in SCCHN tumor cell lines; for comparison, expressions in cell lines representing normal tissue were tested as well. All tumor cell lines represent squamous cell carcinoma (SSC), and all but one were derived from primary tumors. The only exception, XF 354 cells, stem from a lymph node metastasis of an oral SCC. Wang et al. showed a consistent pattern of CCR6 down-regulation and up-regulation of CCR7 in metastatic SCCHN cells (Wang et al. 2005a); however, in the present study, neither CCR6 nor CCR7 expression was detected in XF 354 cells. This result is comparable to a study by Muller et al., reporting no difference in the expression of chemokine receptors in primary SCCHN and corresponding lymph node metastases (Muller et al. 2006).

In general, in the analyzed cell lines, the expression of chemokines was more common than the expression of chemokine receptors. The pronounced chemokine expression by the tumor cell lines may indicate that they reliably mirror the inflammatory microenvironment of tumors, although cultured cancer cells can exhibit distinct properties compared with their naturally growing counterparts. For SCCHN, the (over)-expression and secretion of the CC-chemokines CCL2, CCL5, and CCL20, which were identified in the cell lines of the present study, have repeatedly been described (Abiko et al. 2003; Buettner et al. 2007; Chang et al. 2008; Ferreira et al. 2008; Michiels et al. 2009). The same applies to the expression of the CXC-chemokines, whereupon the homogeneous expression of CXC1-3 is explainable, as they cluster in a narrow region of chromosome 4 and are often coexpressed (Bieche et al. 2007). For SCCHN, CXC-chemokines have been suggested as useful as biomarkers for response and survival in patients with locally advanced oropharyngeal and head and neck SCC treated with radiochemotherapy (Allen et al. 2007).

Of the CXC receptors, only CXCR1 and CXCR4 expression was detected in the analyzed tumor cell lines. Uchida et al. reported on comparable findings using lymph node metastatic (HNt and B88) and non-metastatic oral SCC cells, where of 13 kinds of chemokine receptors examined, only CXCR4 expression was up-regulated (Uchida et al. 2003). Several studies have already documented the importance of CXCR4 in SCCHN tumor progression and organ-specific metastasis (Almofti et al. 2004; Delibasi et al. 2004; Ishikawa et al. 2006; Oliveira-Neto et al. 2008; Rehman and Wang 2008; Tan et al. 2008) and that a high level of CXCR4 could be used as a prognostic factor (Wang et al. 2005c). Furthermore, it was suggested that in cases of oral SCC, the paracrine CXC12/CXCR4 system potentiates lymph node metastasis, but distant metastasis might require the autocrine CXC12/CXCR4 system (Uchida et al. 2007). Interestingly, of the tumor cell lines expressing CXCR4 in the present study, three (HO 1u1, FaDu, ZMK-1) expressed CXCR4 only, and one expressed CXCR4 and its ligand CXC12. The differential expression may render them possible candidates for cell migration and invasion studies after radiation or radiation and drug treatment.

For irradiation, the common clinical single dose of 2 Gy was used, as irradiation at therapeutic doses can induce essential molecular signals required for an effective response of the immune system to the tumor (Demaria and Formenti 2007). Even extremely low-dose ionizing radiation (1 cGy) caused an up-regulation of three CXC-chemokines (CXCL1, CXCL2, and CXCL6) in human fibroblasts (Fujimori et al. 2005). In the present study, the normal cells

![Fig. 2](image-url)

Fig. 2  CXC-chemokine transcript expression relative to housekeeping genes in tumor and normal cell lines; a CXCL1, b CXCL2, c CXCL3. Greater than twofold expression changes were regarded as significant and are marked by asterisks.
Fig. 3 CXC-chemokine transcript expression relative to housekeeping genes in tumor and normal cell lines; a CXCL10, b CXCL11, c CXCL9, d CXCL12. Greater than twofold expression changes were regarded as significant and are marked by asterisks.

Fig. 4 Chemokine receptor transcript expression relative to housekeeping genes in tumor and normal cell lines; a CCR6, b CXCR1, c CXCR4. Greater than twofold expression changes were regarded as significant and are marked by asterisks.
(fibroblasts; HaCat) also showed a radiation-induced up-regulation of CXCL1, CXCL2, and CXCL3, which was always greater than twofold in HaCat cells and greater than twofold for CXCL3 in fibroblasts. We could confirm the CXCL1 expression on the protein level; however, differences between irradiated and non-irradiated cells were marginal. With respect to the CXCL3 mRNA transcript expression, we observed a marked difference between normal and tumor cells; while both normal cell lines showed the above-mentioned radiation-induced up-regulation, a down-regulation was observed in all tumor cell lines.

For further chemokines, e.g., the CC-chemokines CCL2 and CCL5, or the CXC-chemokine CXCL12, transcript expressions were modified after irradiation. It might be worth to test the possibility that chemokines could be used as biomarkers of radiation responses. For mice, it has already been shown that radiation induces earlier and greater temporal changes in multiple cytokines and chemokines in pulmonary fibrosis-sensitive compared to fibrosis-resistant animals (Ao et al. 2009). Herein, we could show persistent expression and significant accumulation of CXCL12 protein in cell culture supernatants of irradiated tumor cells and fibroblasts.

Our study has several limitations. Although a wide panel of cell lines was used, the reported results cannot be generalized but apply solely to the cell lines of the present study. The transcript expressions reported herein and the detection of CXCL1 and CXCL12 proteins in cell culture supernatants are strong indicators of chemokine and chemokine receptor expressions, but will need confirmation by further protein analysis and functional assays in future.

In conclusion, the substantial variation in chemokine and chemokine receptor expression between SCCHN offer opportunities for the establishment of assays to test for the relevance of chemokine and chemokine receptor expression in the response of SCCHN to radiotherapy and radiochemotherapy.

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