Impairment of MLH1 and CDKN2A in oncogenesis of laryngeal cancer

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Our study aimed at elucidating which genetic alterations tend to form a network and could be applied as molecular markers of larynx squamous cell carcinoma (LSCC). A panel of genes involved in tumorigenesis was investigated. To search for the possible mechanisms of gene silencing, loss of heterozygosity (LOH) was analysed followed by testing DNA methylation and protein expression for those genes found with the highest frequency of LOH (CDKN2A (55.4%), MLH1 (46.0%), RB1 (35.7%)). A correlation of both LOH and hypermethylation with the loss of expression for CDKN2A and MLH1 was found. Disrupted Rb pathway (loss of expression of RB1 and/or of CDKN2A) in 55.9% of analysed cases confirmed the hypothesis that RB1 pathway is altered in head and neck squamous cell carcinomas, with CDKN2A (45%), rather than RB1 (11.8%) being more frequently inactivated. In LSCC, LOH tends to occur together in gene pairs or triplets. The pair MLH1/CDKN2A and triplets MLH1/TSG on 8p22/CDKN2A and MLH1/CDKN2A/RB1 are related to staging and grading. LOH in MLH1 correlates with lower and LOH in CDKN2A with higher grades of LSCC. It can be concluded that MLH1 and CDKN2A play an important role in LSCC development and progression.

Keywords: LSCC; CDKN2A (p16); MLH1; RB1

Squamous cell carcinomas of the head and neck (HNSCC) are frequently occurring cancers with a mean incidence of around 5% of all cancers diagnosed worldwide annually. Despite intense efforts to develop early detection methods, as well as effective therapy in HNSCC, the overall 5-year survival rate of 50% remains among the lowest for the major cancers (Papadimitrakopoulou, 2000; Crowe et al, 2002). In the aetiology of HNSCC, both environmental and genetic factors are involved. Here, not only the cancer genes such as oncogenes, tumour suppressor genes (TSGs), mutator genes but also genes that modulate the individual susceptibility to environmental carcinogens, for example, genes involved in xenobiotic metabolism and DNA repair are involved in HNSCC development (Sturgis and Wei, 2002). This makes the genetics of HNSCC extremely complex (Fan, 2001). The situation becomes increasingly complicated since the definition of HNSCC covers a variety of tumours, which differ both in terms of their location within the upper aerodigestive tract and also in histopathology. Successive publications revealed the involvement of a variety of genes in the development and progression of HNSCC, such as oncogenes, for example, MYC, RAS, ERBB2, BCL2, INT2 and TSG, for example, TP53, CDKN2 (Voravud et al, 1997). It was also shown that a poor clinical course of HNSCC is linked to loss of heterozygosity (LOH) in multiple loci (Gleich et al, 1999), especially to allelic loss at 3p, 8q, 13q and 17p (Li et al, 1994; Scholnick et al, 1996; El-Naggar et al, 1997), while LOH at 9p21 and 17q31 is related to a high incidence of recurrent tumours (Matsuura et al, 1998). The karyotypes in HNSCC were shown to be complex with breakpoints underlying chromosomal alterations located mainly at 1p, 1q, 3p, 3q, 4q, 8p, 8q, 9p, 10p, 10q, 11q, 13p, 14p, 15p and 15q. Nonrandom patterns of chromosomal aberrations in the progression of HNSCC have been suggested (Jin et al, 1990; Van Dyke et al, 1994; Soder et al, 1995). Isochromosomes 8q, deletion 3p and homogenously staining regions at 11q13 were most often observed among the recurrent structural chromosomal aberrations (Van Dyke et al, 1994; Soder et al, 1995; Bergamo et al, 2000). The application of comparative genomic hybridisation (CGH) permitted the identification of chromosomal imbalances (Kujawski et al, 1999; Bergamo et al, 2000). The gains were observed mainly in 3q and 8q, while losses occurred in 3p and 22q. Also, a correlation between some clinical data and chromosomal alterations was reported, such as association between the gains of 1q and 2q and a refractory clinical response (Bergamo et al, 2000), and more frequent losses at 13q, 8p and 9q in metastatic than in primary tumours (Kujawski et al, 1999).

Although abundant molecular and cytogenetic data on HNSCC have been collected, neither critical genes nor a generally accepted genetic model of HNSCC development and progression have been described. The first model suggested for HNSCC combines, as an early event, multiple LOH with allelic loss at 9p, 3p, 17q, 4q and 13q. LOH at 18q and 8p are considered late genetic events (Califano et al, 1996). On the basis of already published cytogenic

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data, two main genetic pathways in HNSCC were suggested (Höglund et al., 2001) by using the two principal components of genomic imbalance (gains and losses): one with −1p, −1q and −7q as an early event followed by −8p and −4p, and another starting with +7q, and subsequently followed by +11p, +8q and +1p. Both then converge to a common set of imbalances: −3p, −9p and −11q. In the recently published ‘oncogenic trees’ for HNSCC progression, three subsets of tumours, which differed in their localisation (pharyngeal, laryngeal and oral squamous cell carcinoma), were analysed separately by using CGH (Huang et al., 2002). The authors proposed that generally, +3q/−3p is the most important chromosomal event in the genetic aetiology of HNSCC, which then may be followed by other chromosomal imbalances, occurring with various frequencies depending on cancer location.

Despite the lack of precise genetic information, it seems clear that HNSCC results from the accumulation of changes in genes controlling genomic stability, proliferation, apoptosis and invasion, and that these changes tend to form a functional network. Therefore, we decided to investigate the interconnections of genes involved in different pathways, which were observed to be most often altered in cancers. The rationale underlying the choice of genes to be tested was based on recently published results. Our collection consisted of tumour suppressors such as TP53, CDKN2A, RB1, which represent the most common type of HNSCC. The sequences for all primers are listed in the Genome Database (http://gdbwww.gdb.org).

Materials and Methods

Samples

To analyse the homogeneous set of primary LSCC, cancer tissues and matched blood samples were obtained from 62 patients diagnosed with the primary LSCC in the Department of Otolaryngology, Wroclaw Medical University, Poland. All the tumours were diagnosed histopathologically according to the World Health Organisation’s criteria as carcinoma planoepitheliale keratodes (33 cases), akeratodes (25 cases) and keratoblasticum (four cases). Biological material for molecular analysis was collected before chemotherapy and/or radiotherapy. Cancer tissues for molecular analyses were dissected from the central part of tumour. None of the patients had a history of hereditary cancer.

DNA amplification and LOH analysis

DNA was isolated from cancer tissue and corresponding peripheral blood lymphocytes following standard procedures. Extracted DNA (3 μg) was DOP-amplified (Degenerate Oligonucleotide Primed-PCR® with the NRich-kit, Genpak, UK) following the manufacturer’s guidelines. To test for genes important in carcinogenesis, a panel of microsatellite markers was applied: D1S2883, D2S123, D3S1611, D5S346, D8S254, NME1, NME2, NME3, TP53, TFF1, TFF2, D9S171 and RB1. The sequences for all primers are listed in the Genome Database (http://gdbwww.gdb.org). The LOH was

| Age | Sex | Histopathology | Grading | T | N | M | Staging |
|-----|-----|----------------|---------|---|---|---|---------|
| 59  | M   | k              | 2       | 3 | 2 | 0 | 4a      |
| 48  | F   | k              | 3       | 4 | 0 | 0 | 4a      |
| 54  | M   | k              | 2       | 4 | 0 | 0 | 4a      |
| 54  | M   | k              | 2       | 3 | 2 | 0 | 4a      |
| 57  | M   | k              | 2       | 4 | 0 | 0 | 4a      |
| 59  | M   | k              | 1       | 4 | 0 | 0 | 4a      |
| 64  | M   | k              | 2       | 4 | 1 | 0 | 4a      |
| 50  | M   | ak             | 2       | 3 | 0 | 3 |        |
| 62  | M   | k              | 1       | 4 | 1 | 0 | 4a      |
| 66  | M   | k              | 2       | 4 | 0 | 0 | 4a      |
| 65  | M   | ak             | 2       | 3 | 0 | 0 | 3       |
| 45  | M   | ak             | 2       | 4 | 1 | 0 | 4a      |
| 61  | M   | k              | 1       | 1 | 0 | 0 | 1       |
| 63  | M   | k              | 2       | 3 | 0 | 3 |        |
| 60  | M   | k              | 3       | 4 | 0 | 0 | 4a      |
| 67  | M   | k              | 2       | 3 | 0 | 0 | 3       |
| 62  | M   | k              | 2       | 3 | 0 | 0 | 3       |
| 64  | M   | ak             | 1       | 3 | 0 | 0 | 3       |
| 56  | M   | ak             | 3       | 3 | 0 | 0 | 3       |
| 68  | M   | ak             | 3       | 4 | 3 | 0 | 4b      |
| 54  | M   | ak             | 2       | 4 | 0 | 0 | 4a      |
| 71  | M   | kb             | 2       | 4 | 1 | 0 | 4a      |
| 60  | M   | k              | 3       | 3 | 0 | 0 | 3       |
| 64  | F   | ak             | 2       | 4 | 0 | 0 | 4a      |
| 59  | M   | k              | 1       | 3 | 0 | 0 | 3       |
| 69  | M   | ak             | 3       | 1 | 0 | 0 | 1       |
| 51  | M   | k              | 2       | 3 | 0 | 0 | 3       |
| 44  | M   | k              | 2       | 2 | 0 | 0 | 2       |
| 57  | M   | ak             | 2       | 3 | 0 | 0 | 3       |
| 61  | M   | ak             | 3       | 3 | 0 | 0 | 3       |
| 48  | M   | kb             | 2       | 3 | 1 | 0 | 3       |
| 61  | F   | ak             | 3       | 3 | 2 | 0 | 4a      |
| 69  | F   | ak             | 3       | 4 | 2 | 0 | 4a      |
| 56  | M   | ak             | 2       | 3 | 2 | 0 | 4a      |
| 43  | M   | ak             | 2       | 3 | 0 | 0 | 3       |
| 50  | M   | ak             | 2       | 3 | 0 | 0 | 3       |
| 67  | M   | kb             | 2       | 3 | 0 | 0 | 3       |
| 58  | M   | ak             | 3       | 3 | 0 | 0 | 3       |
| 63  | M   | kb             | 2       | 4 | 0 | 0 | 4a      |
| 71  | M   | ak             | 3       | 1 | 0 | 0 | 1       |
| 60  | M   | k              | 2       | 1 | 0 | 0 | 1       |
| 71  | M   | k              | 2       | 4 | 0 | 0 | 4a      |
| 50  | M   | k              | 1       | 3 | 0 | 0 | 3       |
| 49  | M   | k              | 1       | 2 | 0 | 0 | 2       |
| 69  | M   | ak             | 3       | 4 | 0 | 0 | 4a      |
| 61  | M   | k              | 2       | 3 | 1 | 0 | 4a      |
| 60  | M   | ak             | 2       | 3 | 0 | 0 | 3       |
| 49  | M   | ak             | 1       | 4 | 1 | 0 | 4a      |
| 61  | F   | k              | 3       | 3 | 0 | 0 | 3       |
| 51  | M   | ak             | 3       | 4 | 2 | 0 | 4a      |
| 60  | M   | ak             | 3       | 3 | 0 | 0 | 3       |
| 46  | M   | ak             | 2       | 3 | 0 | 0 | 3       |
| 57  | F   | ak             | 2       | 2 | 0 | 0 | 2       |
| 48  | M   | k              | 2       | 3 | 0 | 0 | 3       |
| 51  | M   | k              | 2       | 4 | 0 | 0 | 4a      |
| 61  | M   | k              | 1       | 3 | 0 | 0 | 3       |
| 64  | M   | k              | 2       | 4 | 1 | 0 | 4a      |
| 51  | M   | k              | 2       | 4 | 2 | 0 | 4a      |
| 49  | M   | k              | 1       | 4 | 0 | 0 | 4a      |
| 55  | M   | k              | 1       | 4 | 0 | 0 | 4a      |
| 52  | M   | k              | 1       | 4 | 0 | 0 | 4a      |
| 56  | F   | k              | 2       | 4 | 0 | 0 | 4a      |

F = female; M = male; k = carcinoma planoepitheliale keratodes; ak = carcinoma planoepitheliale akeratodes; kb = carcinoma planoepitheliale keratoblasticum; LSCC = larynx squamous cell carcinoma.
study in larynx carcinoma DNA and in matched constitutional DNA from the original series. PCR was performed according to the standard protocol in a PTC-200 thermocycler (MJ Research, USA). Fluorescent PCR products were pooled and resolved on a 4% polyacrylamide gel supplemented with 7 M urea in an ABI-377 sequencing device. Cold PCR amplification of TFF1 and TFF2 was performed as described previously (Carvalho et al., 2002). Sizes and quantity of marker alleles were evaluated by a semiautomated analysis using GENESCAN software, version 3.1, and the GENOTYPER software package, version 2.0 (Applied Biosystems, USA). Allelic loss was defined as a more than 70% reduction in the tumour peak area compared to the peak area of corresponding normal tissue (Karnik et al., 1998).

PCR with methylation-specific primers (MSP-PCR) was used to assess the promoter methylation of the CDKN2A. Genomic DNA was bisulphite treated followed by amplification performed by cold PCR as described previously using the following primers: forward, 5’-attagtggagatttgtttaga-3’; reverse, 5’-aaaaaataccatctacctatct-3’ (Herman et al., 1996). Cycling conditions were 30 s at 94°C, 30 s at 55°C and 30 s at 72°C for 35 cycles. Methylation of the MLH1 promoter regions was examined using previously described digestion protocols with some modifications. Normal and tumour DNA were digested using HpaII and MspI enzymes. The MLH1 promoter region was analysed using two pairs of specific primers: MLH1-881 and MLH1-1219 or MLH1-881 and MLH1-1470 described elsewhere. (Papadimitrakopoulou, 2000; Rainho et al., 2001; Fiedler et al., 2002) PCR products were separated in nondenatured 6% PAA gels, stained in ethidium bromide and directly visualised by UV illumination.

Immunostaining

Immunostaining for MLH1, P16 and RB was performed using the streptavidin–biotin peroxidase method according to standard procedures. The samples were immunostained using the following monoclonal antibodies: MLH1 IgG1 (clone G168-15, BD Biosciences, Germany), P16 IgG2a (clone F-12, sc-1661, Santa Cruz Biotechnology Inc., USA) and RB IgG1 (clone RB1 1F8, Dako, Denmark) in the dilution 1:400, 1:50 and 1:100, respectively. Specimens were counterstained with haematoxylin and analysed in a light microscope. Normal human laryngeal tissue was used as a negative control. The number of positive cells per high field was assessed. The immunoreactivity results were recorded as positive when at least 20% of nuclear cells were stained positively, and any reduction below 20% in the number of stained cells was considered as an abnormal pattern.

Table 2 Results of analysis of LOH, methylation and protein expression in LSCC

| Gene       | Chromosomal localisation | LOH | Aberrant methylation | Decreased expression |
|------------|--------------------------|-----|----------------------|----------------------|
| MLH1       | 3p22                     | 23/50 (46.0) | 14/62 (22.6) | 14/51 (27.5) |
| TSG        | 8p22                     | 21/55 (38.0) | 7/59 (11.8) | 27/60 (45) |
| RB1        | 13q14                    | 20/56 (35.7) | 21/56 (37.5) | 7/59 (11.8) |
| CDKN2A HPC1| 9p21                     | 31/56 (55.4) | 37/56 (67.5) | 21/36 (35) |
| APC        | 1q24                     | 6/44 (13.6)  | 10/60 (16.6) | 7/49 (14.2) |
| MSH2       | 5q21                     | 10/60 (16.6) | 14/62 (22.6) | 14/51 (27.5) |
| TP53       | 2p16                     | 7/49 (14.2)  | 14/62 (22.6) | 14/51 (27.5) |
| NME1       | 17p13                    | 4/51 (7.8)   | 7/59 (11.8)  | 27/60 (45)  |
| NME2       | 17q21                    | 12/57 (21.0) | 21/56 (37.5) | 37/56 (67.5) |
| NME3       | 17q21                    | 4/43 (9.3)   | 14/62 (22.6) | 14/51 (27.5) |
| TFF1       | 17q21                    | 7/54 (12.9)  | 7/59 (11.8)  | 27/60 (45)  |
| TFF2       | 21q22.3                  | 2/19 (10.5)  | 14/62 (22.6) | 14/51 (27.5) |

*The percentage was calculated for the informative cases. aLOH, loss of heterozygosity; LSCC = larynx squamous cell carcinoma. bTSG (unknown tumour suppressor gene on 8p22).
a significant value (P<0.01, Spearman’s test). However, no statistical significance was discernible between the LOH and loss of protein expression for the RB1 locus. The following correlations of LOH, methylation and loss of protein expression with tumour grading were observed: negative for MLH1 and positive for CDKN2A (Table 3).

To search for genetic alterations that tend to occur together to form a network of molecular events, Spearman’s test and association analysis were applied. We noted that in the following genes tends to occur in pairs: TSG on 8p22/NME1, MLH1/CDKN2A (P<0.01, Spearman’s test) and with lower statistical significance in TSG on 8p22/MLH2, TSG on 8p22/NME3, MSH2/NME2, MSH2/APC (P<0.05, Spearman’s test; Table 4).

Analysis of the LOH pairs mentioned above and clinicohistopathological features of disease showed a positive correlation for only one pair: MLH1/CDKN2A and staging (P<0.05, ANOVA) and grading (P<0.01, ANOVA). For this pair, LOH in MLH1 correlates negatively but LOH in CDKN2A correlates positively with grading (P<0.01, ANOVA). Further application of association analysis for triple parameters indicated a correlation for the following sets: MLH1/CDKN2A/TSG on 8p22 and MLH1/CDKN2A/RB1 (Table 4). Detailed statistical analysis showed that in both triplets, LOH in MLH1 correlates with lower, and in CDKN2A with higher grading (P<0.01, ANOVA), but LOH in TSG on 8p22 and RB1 gene are not directly linked to tumour grading.

DISCUSSION

Analysis of allelic loss (LOH) is widely applied in searching for tumour suppressor genes involved in the process of neoplastic transformation. The analysis of LOH indicated the involvement of a variety of genes in the development and progression of LSCC (Rainho et al, 2001; dos Reis et al, 2002; Fiedler et al, 2002; Gunduz et al, 2002). It is hypothesised that LSCC development is the result of the accumulation of six to 10 independent genetic events (Renan, 1993). Therefore, our study focused on searching for alterations in a chosen panel of genes reported to be frequently altered in manifold cancers. In our series of LSCCs, LOH was most frequently observed in microsatellites linked to the following genes: CDKN2A (55.4%), MLH1 (46.0%), TSG on 8p22 (38%), RB1 (35.7%) and NME1 (21%). The function of some of them in tumorigenesis is well known. CDKN2A and RB1 play an important role in the cell cycle control (in RB pathway) (Sherr, 1996; Yokoyama et al, 1996). MLH1 belongs to the group of genes controlling mismatch repair (Deng et al, 1999; Wheeler et al, 1999; Arzimanoglou et al, 2002). However, the location of putative genes on 8p and their association with the development and progression of HNSCC are still disputed (Li et al, 1994; Nawroz et al, 1994; Fujitaka et al, 1995).

Therefore, we chose CDKN2A, RB1 and MLH1 for a more detailed molecular analysis. Since promoter methylation following LOH is frequently involved in the silencing of CDKN2A and MLH1 (El-Naggar et al, 1997; Deng et al, 1999; Wheeler et al, 1999), but not RB1 (Yokoyama et al, 1996; Gleich et al, 1999), the analysis of methylation of CDKN2A and MLH1 was also performed. The positive correlation of both LOH and hypermethylation with loss of protein expression for CDKN2A and MLH1 genes (P<0.01) confirmed the thesis that these are the most important mechanisms for silencing the CDKN2A and MLH1 genes (El-Naggar et al, 1997; Deng et al, 1999; Wheeler et al, 1999). Our results, showing impaired Rb pathway (loss of expression of RB1 and/or of CDKN2A) in 55.9% of analysed cases confirmed the hypothesis that RB1 pathway (55.9%) is commonly disrupted in HNSCC development and progression, with CDKN2A (45%), rather than RB1 (11.8%) being the frequent direct target for inactivation (Lang et al, 2002). Despite the fact that in our series downregulation of MLH1 was observed in 27.5% of cases, analysis of microsatellite instability (MSI) by using BAT 25, BAT 26 and BAT 40 markers showed only low-frequency MSI (MSI-L) in three out of 62 analysed cases (published elsewhere) (Sasiadek et al, 2002). Therefore, we concluded that in LSCC inactivation of MLH1 does not lead to MSI, in contrast to the observation for hereditary nonpolyposis colon cancer (Wheeler et al, 1999). A similar observation was reported by Arzimanoglou et al, 2002), who observed frequent LOH at MLH1 and negligible DNA instability in ovarian cancer. These results support the hypothesis that microsatellite stability is controlled by a variety of genes (Giannini et al, 2002). Statistical analysis of LOH in MLH1, CDKN2A and RB1 genes and clinicohistopathological features of the disease disclosed that LOH in MLH1 and CDKN2A correlates only with tumour grading. Our results suggest that LOH in MLH1 is characteristic for lower, while LOH in CDKN2A occurs in higher grades of LSCC (Table 3). We searched for the significance of combinations of LOH in two or three loci taking into account the opinion of Huang et al (2002) that sole analysis of single genetic alterations may neglect the important role of a combination of two or more alterations during the progression of cancer. We found six pairs and two triplets of genes in which LOH tends to occur together. The analysis of their correlation with clinicohistopathological features of the disease proved that one pair (MLH1/CDKN2A) and both triplets are related to staging and grading. We observed that

| Grade | LOH (%) | Aberrant methylation (%) | Decreased expression (%) | LOH (%) | Aberrant methylation (%) | Decreased expression (%) |
|-------|---------|--------------------------|--------------------------|---------|--------------------------|--------------------------|
| 1     | 45.5    | 27.27                    | 50                       | 62.5    | 33.33                    | 41                       |
| 2     | 47.05   | 32.25                    | 33.33                    | 50      | 27.7                     | 26.9                     |
| 3     | 83.33   | 57.14                    | 66.66                    | 30      | 6.6                      | 0                        |

*The percentage was calculated for the informative cases. **LOH; loss of heterozygosity; LSCC = larynx squamous cell carcinoma. Statistics significant results – P<0.01; ANOVA. *Statistically significant results – P<0.05; ANOVA.
in each of these cases LOH in MLH1 correlates with lower and LOH in CDKN2A with higher grades of LSCC. Similar correlations were observed in the analysis of LOH in single loci. Therefore, it can be postulated that MLH1 and CDKN2A play an important role in LSCC development and progression.

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