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M6P/IGF2R loss of heterozygosity in head and neck cancer associated with poor patient prognosis

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Abstract

Background: The *mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R)* encodes for a multifunctional receptor involved in lysosomal enzyme trafficking, fetal organogenesis, cytotoxic T cell-induced apoptosis and tumor suppression. The purpose of this investigation was to determine if the *M6P/IGF2R* tumor suppressor gene is mutated in human head and neck cancer, and if allelic loss is associated with poor patient prognosis.

Methods: *M6P/IGF2R* loss of heterozygosity in locally advanced squamous cell carcinoma of the head and neck was assessed with six different gene-specific nucleotide polymorphisms. The patients studied were enrolled in a phase 3 trial of twice daily radiotherapy with or without concurrent chemotherapy; median follow-up for surviving patients is 76 months.

Results: *M6P/IGF2R* was polymorphic in 64% (56/87) of patients, and 54% (30/56) of the tumors in these informative patients had loss of heterozygosity. *M6P/IGF2R* loss of heterozygosity was associated with a significantly reduced 5 year relapse-free survival (23% vs. 69%, p = 0.02), locoregional control (34% vs. 75%, p = 0.03) and cause specific survival (29% vs. 75%, p = 0.02) in the patients treated with radiotherapy alone. Concomitant chemotherapy resulted in a better outcome when compared to radiotherapy alone only in those patients whose tumors had *M6P/IGF2R* loss of heterozygosity.

Conclusions: This study provides the first evidence that *M6P/IGF2R* loss of heterozygosity predicts for poor therapeutic outcome in patients treated with radiotherapy alone. Our findings also indicate that head and neck cancer patients with *M6P/IGF2R* allelic loss benefit most from concurrent chemotherapy.

Background

Squamous cell carcinoma of the head and neck is diagnosed in over 40,000 Americans each year, resulting in over 12,000 annual deaths [1]. Carcinomas of the head and neck are often associated with multiple areas of dysplasia or carcinoma *in situ* (CIS) in noncontiguous mucosa, as well as with the development of second primary cancers of the aerodigestive tract. The concept of "field cancerization" hypothesizes that regions of the mucosal epithelium, although normal in appearance, are "preconditioned" by chronic exposure to carcinogenic agents, thus priming them for the subsequent development of invasive lesions [2].

The use of modern molecular biological techniques has supported and greatly expanded our understanding of the "cancer field effect". Analysis of X-chromosome inactivation in female patients with multiple head and neck cancers shows that distinct tumors arise from regional clonal growths of phenotypically normal, mutated preneoplastic cells [3–5], a phenomenon similar to that also observed in liver cancer patients with cirrhosis [6]. Long-term smoking and alcohol abuse are strongly associated with these clonal growths in the upper aerodigestive tract (reviewed in [7]).

Frequent allelic loss at chromosomal locations 2q, 3p, 4q, 6p, 6q, 8p, 8q, 9p, 11q, 13q, 14q and 17q is observed in head and neck cancer [4,8–11]. Molecular studies of hyperplastic, dysplastic, CIS, and invasive head and neck lesions indicate that loss of heterozygosity at chromosomal locations 3p, 9p and 17p are early events in head and neck carcinogenesis [4,12]. Specific tumor suppressor genes, such as *FHIT* (3p14.2) *p16* (9p21), *p53* (17p13.1) and *RB1* (13q14.2) have also been shown to be mutated in head and neck cancer [4,13]. Nevertheless, the large number of additional chromosomal regions with high frequencies of allelic loss in head and neck cancer strongly suggests that many suppressor genes still remain to be identified.

The *mannose 6-phosphate/insulin-like growth factor 2 receptor (M6P/IGF2R)* maps to chromosome location 6q25-27 [14]; a chromosomal region predicted to contain a head and neck tumor suppressor gene [10,11]. It encodes for a receptor that functions in intracellular lysosomal enzyme trafficking, transforming growth factor beta activation, and IGF2 degradation (reviewed in [15,16]). Granzyme B internalization by the M6P/IGF2R is also required for cytotoxic T cells to induce apoptosis in cells targeted for death, resulting in this receptor being referred to as a "death receptor" [17]. Elevated IGF2 levels during murine development arising from M6P/IGF2R deficiency result in cardiac abnormalities, cleft palate, fetal overgrowth and perinatal lethality [15,18]. Furthermore, large offspring syndrome frequently observed in cloned animals is associated with epigenetic changes in gene regulation and decreased *M6P/IGF2R* expression [19]. Thus, the *M6P/IGF2R* plays a crucial role in regulating mammalian fetal growth and development.

The *M6P/IGF2R* is also mechanistically involved in the genesis of human cancer [6,20–24]. *M6P/IGF2R* loss of heterozygosity coupled with intragenic loss-of-function mutations in the remaining allele is a common event in human cancers [6,20–22]. Inheritance of a tandem repeat polymorphism in the 3' UTR of *M6P/IGF2R* furthermore predicts for enhanced susceptibility to oral cancer [25]. Moreover, tumor cell growth is inhibited when *M6P/IGF2R* expression is restored to normal while it is increased when gene expression is reduced [26–29]. The results of these mutational and functional studies clearly demonstrate that the *M6P/IGF2R* possesses the characteristics necessary to be classified as a tumor suppressor gene [30].

We tested in this investigation the hypothesis that *M6P/IGF2R* loss of heterozygosity in locally advanced, non-metastatic squamous cell carcinomas of the head and neck is associated with poorer patient prognosis. The patients used in this study were enrolled in a randomized trial of twice daily radiotherapy with or without concurrent chemotherapy [31]. We report herein that *M6P/IGF2R* loss of heterozygosity occurs frequently in head and neck cancer, and that it predicts for poor therapeutic outcome.

Methods

Patient Population and Treatment

Patients with locally advanced but non-metastatic squamous carcinoma of the head and neck who participated in or who met eligibility criteria but declined enrollment in a phase 3 trial of hyperfractionated irradiation with or without concurrent chemotherapy constituted the study population. Details of their treatment and outcome have been published previously [31]. Briefly, patients receiving radiation therapy alone were given a continuous course of 125 cGy twice daily to a total dose of 75 Gy. Patients assigned to combined modality treatment received 70 Gy via a split-course of 125 cGy twice daily. They also received two cycles of cisplatin and 5-fluorouracil during the first and sixth weeks of irradiation. All protocols were approved by the Duke University Medical Center Institutional Review Board.

Tissue Microdissection and M6P/IGF2R *Loss of Heterozygosity Analysis*

Microdissection of malignant and surrounding normal non-mucosal tissue was performed as previously described [6,20,21]; tumor histology was confirmed by a pathologist (R.T. Volmer). Paraffin-embedded sections (10

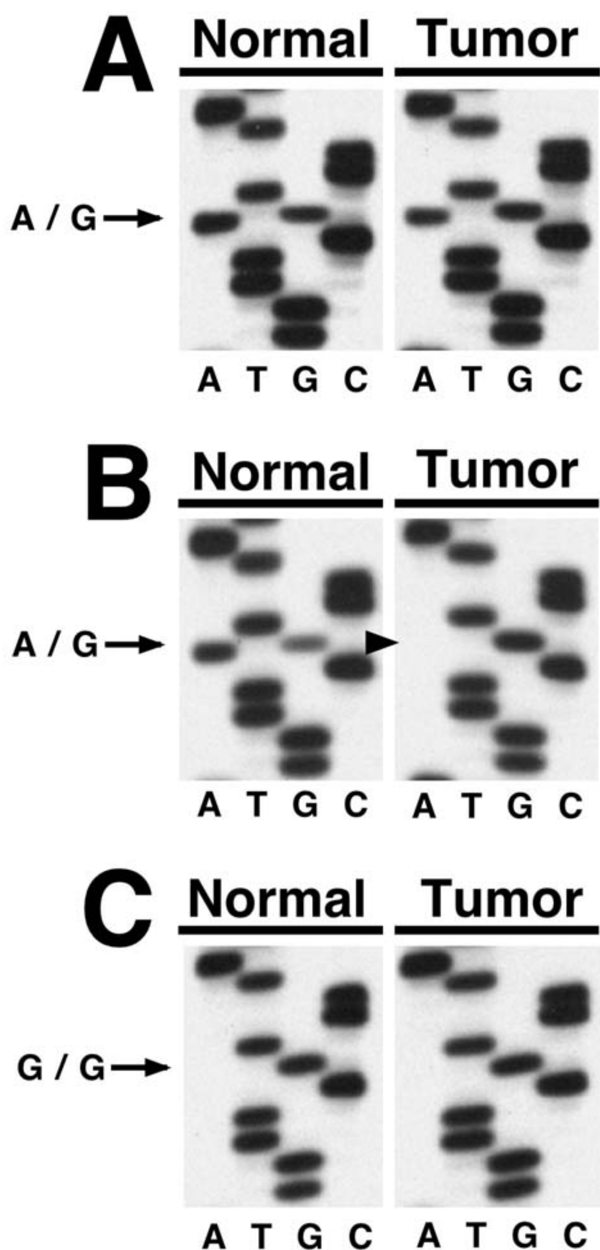

Figure 1
M6P/IGF2R loss of heterozygosity in human head and neck cancer. A single nucleotide polymorphism (c. 1197A>G transition) in exon 9 of the *M6P/IGF2R* was used to determine loss of heterozygosity in these representative samples [32,33]. **(A)** Informative head and neck cancer without *M6P/IGF2R* loss of heterozygosity (i.e. both A and G polymorphisms expressed in tumor). **(B)** Informative head and neck cancer with *M6P/IGF2R* loss of heterozygosity (i.e. only G polymorphism expressed in tumor). **(C)** Non-informative head and neck cancer. Arrow, location of A>G polymorphism; arrowhead, A allele absent.

μm in thickness) were microdissected following deparaffinization, and the tissue was then digested by proteinase K in tris-EDTA buffer at 55°C for 4 hr. Six different gene-specific polymorphisms were used to assess head and neck tumors for *M6P/IGF2R* loss of heterozygosity [32,33].

A tetranucleotide (ACAA) insertion/deletion polymorphism in the 3'-UTR of the *M6P/IGF2R* [34] was detected with 2 rounds of nested polymerase chain reaction (PCR). The forward and reverse first round PCR primers were KK*ACAA*F1: 5'-GGAGAGTTTGCCTGTTCTATGCC-3' and KK*ACAA*R: 5'-CAAATCAATCTTTGGGC AGG-3', respectively. The forward and reverse second round PCR primers were KK*ACAA*F2: 5'-AGTCAGGAATGGCT-GCACC-3'and KK*ACAA*R: 5'-CAAATCAATC TTT-GGGCAGG-3', respectively; the KK*ACAA*F2 primer was end-radiolabeled with ^{33}P-dATP prior to PCR. Each round of PCR consisted of 31 cycles at 94°C for 20 seconds, 55°C for 40 seconds, and 72°C for 45 seconds; Platinum® Taq DNA polymerase (GibcoBRL, Baltimore, MD) was used for DNA amplification. The PCR product (5 μl) containing the 3'-UTR (ACAA) insertion/deletion polymorphism was mixed with 5 μl of formamide-based stop buffer, heated to 95°C for 10 min, placed on ice, electrophoresed on a 6% acrylamide gel, and then exposed to film.

Five identified single nucleotide polymorphisms, c. 901C>G (exon 6), c. 1197A>G (exon 9), c. 1737A>G (exon 12), c. 2286A>G (exon 16), and c. 5002A>G (exon 34), were also analyzed following 2 rounds of nested PCR; the exon specific forward and reverse primers have been previously described [32,33]. The exons containing these polymorphisms were PCR amplified from genomic DNA using PCR conditions identical to those described above. The single nucleotide polymorphisms used to determine *M6P/IGF2R* loss of heterozygosity were assessed by direct sequencing of PCR products according to the manufacturer's protocol (Thermo Sequenase, USB Corporation, Cleveland, OH) (Figure 1). *M6P/IGF2R* loss of heterozygosity frequency is independent of the polymorphism used for its estimation ($p = 0.3$).

Taq DNA polymerase can introduce sequence errors during PCR amplification, and unequal amplification of the two alleles can result in false positive detection of loss of heterozygosity. Thus, both the normal and tumor DNA templates were amplified in three independent PCR reactions, and assessed for tumor *M6P/IGF2R* loss of heterozygosity. Due to the potential of contaminating the tumor tissue sample with normal stroma, allele loss in informative patients was defined as a >50% decrease in the ratio of the polymorphic band intensities in the tumor tissue versus that in the surrounding normal stromal tissue; this was quantified using a densitometer.

Table 1: Clinical characteristics of head and neck cancer patients

Characteristics	**Mutated *M6P/IGF2R*** (n = 30)	**Non Mutated *M6P/IGF2R*** (n = 26)	**Informative Patients** (n = 56)	**Excluded Patients** (n = 83)
Gender (No.)	Male: 25 (83%)	Male: 23 (89%)	Male: 48 (86%)	Male: 66 (80%)
p = 0.7[1]; p = 0.4[2]	Female: 5 (17%)	Female: 3 (11%)	Female: 8 (14%)	Female: 17 (20%)
Age (Years)	Median: 60.4	Median: 60.9	Median: 61	Median: 60
p = 1.0; p = 0.5	Range: 37–71	Range: 39 – 74	Range: 37 – 74	Range: 30 – 75
Race (No.)	White: 25 (83%)	White: 17 (65%)	White: 42 (75%)	White: 52 (63%)
p = 0.2; p = 0.3	Black: 5 (17%)	Black: 8 (31%)	Black: 13 (23%)	Black: 30 (36%)
		Native American: 1 (4%)	Native American: 1 (2%)	Native American: 1 (1%)
Smoking History	Yes: 30 (100%)	Yes: 23 (89%)	Yes: 53 (95%)	Yes: 76 (92%)
p = 0.1; p = 0.5	No: 0 (0%)	No: 3 (11%)	No: 3 (5%)	No: 7 (8%)
Baseline Hgb (g/dL)	Median: 14.2	Median: 14	Median: 14.2	Median: 13.5
p = 0.8; p = 0.06	Range: 11.5 – 17	Range: 9.9 – 16.9	Range: 9.9 – 17.3	Range: 10.8 – 25
Karnofsky Performance	Median: 90	Median: 90	Median: 90	Median: 90
Status p = 0.9; p = 0.2	Range: 60–100	Range: 70 – 100	Range: 60 – 100	Range: 60 – 100
Tumor Resectability	Yes: 18 (60%)	Yes: 14 (53%)	Yes: 32 (57%)	Yes: 49 (59%)
p = 0.8; p = 0.5	No: 12 (40%)	No: 12 (47%)	No: 24 (43%)	No: 34 (41%)
Follow-up (Months)	Median: 29	Median: 52	Median: 38.6	Median: 28.2
	Range: 2 – 100	Range: 3–124	Range: 2 – 125	Range: 2 – 97
Treatment (No.)				
p = 0.8; p = 0.8				
RT alone	17 (57%)	16 (61%)	33 (59%)	46 (55%)
RT + Chemotherapy	13 (43%)	10 (39%)	23 (41%)	37 (45%)
Tumor Stage (No.)				
p = 0.4; p = 0.3				
T1	0	0	0	1 (1%)
T2	3 (10%)	6 (23%)	9 (16%)	6 (8%)
T3	14 (47%)	11 (42%)	25 (45%)	45 (54%)
T4	13 (43%)	9 (35%)	22 (39%)	30 (36%)
Tx	0	0	0	1 (1%)
Nodal Stage (No.)				
p = 0.6; p = 0.2				
N0	9 (30%)	8 (31%)	17 (30%)	24 (29%)
N1	7 (23%)	3 (12%)	10 (18%)	15 (18%)
N2	12 (40%)	14 (53%)	26 (46%)	30 (36%)
N3	2 (7%)	1 (4%)	3 (5%)	14 (17%)
Overall Staging (No.)				
p = 0.4; p = 0.08				
II	0	2 (8%)	2 (4%)	0
III	11 (37%)	7 (27%)	18 (32%)	24 (29%)
IVa	17 (57%)	16 (61%)	33 (59%)	45 (54%)
IVb	2 (7%)	1 (4%)	3 (5%)	14 (17%)
Primary Tumor Site (No.)				
p = 0.1; p = 0.07				
Base of tongue	7 (23%)	10 (38%)	17 (30%)	15 (18%)
Tonsil	7 (23%)	4 (16%)	11 (20%)	23 (27%)
Larynx	9 (30%)	3 (11%)	12 (21%)	11 (13%)
Hypopharynx	3 (10)	6 (23%)	9 (16%)	17 (21%)
Paranasal Sinuses	0	2 (8%)	2 (4%)	5 (6%)
Oral Cavity	4 (14%)	1 (4%)	5 (9%)	3 (4%)
Nasopharynx	0	0	0	8 (10%)
Unknown	0	0	0	1 (1%)

[1]p-value for comparison between patients with *M6P/IGF2R* mutated and non mutated tumors. [2]p-value for comparison between the *M6P/IGF2R* informative patients and excluded patients.

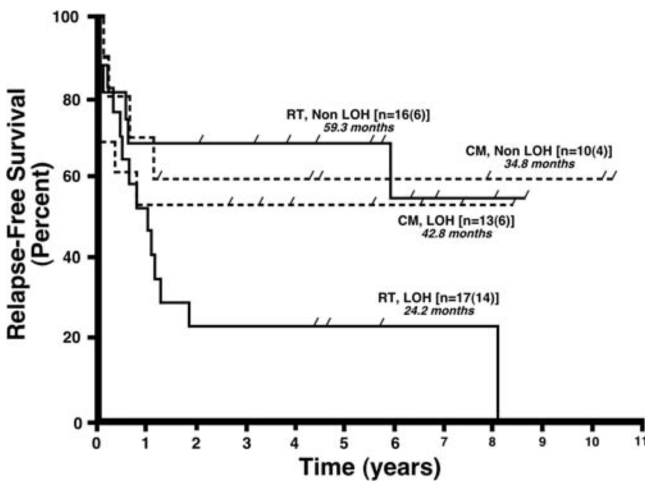

Figure 2
Kaplan-Meier estimates of relapse-free survival in patients with head and neck cancer. Tumors treated with radiotherapy alone (solid lines) were either mutated (RT, LOH) or unmutated (RT, non LOH) at the *M6P/IGF2R* locus. Tumors treated with combined modality therapy (dashed lines) were also either mutated (CM, LOH) or unmutated (CM, non LOH) at the *M6P/IGF2R* locus. Patient number (n), patients that failed (parentheses), censored patients (angled bars), and median follow-up times are provided for each patient group. RT, radiotherapy; CM, combined modality therapy; LOH, loss of heterozygosity.

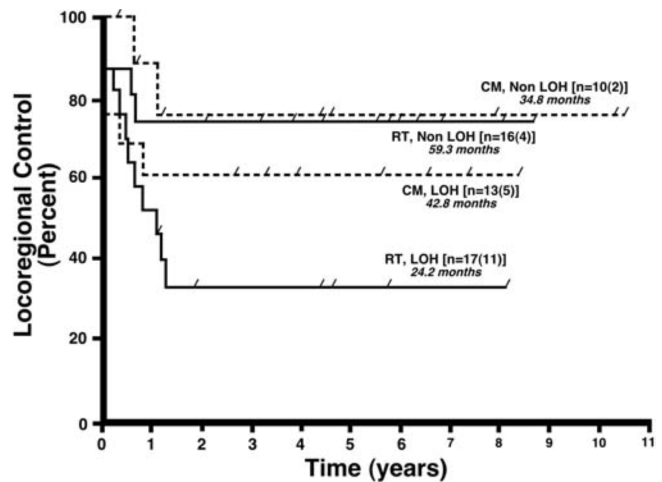

Figure 3
Kaplan-Meier estimates of locoregional control in patients with head and neck cancer. Tumors treated with radiotherapy alone (solid lines) were either mutated (RT, LOH) or unmutated (RT, non LOH) at the *M6P/IGF2R* locus. Tumors treated with combined modality therapy (dashed lines) were also either mutated (CM, LOH) or unmutated (CM, non LOH) at the *M6P/IGF2R* locus. Patient number (n), patients that failed (parentheses), censored patients (angled bars), and median follow-up times are provided for each patient group. RT, radiotherapy; CM, combined modality therapy; LOH, loss of heterozygosity.

Statistical Analysis

Relapse-free survival represented the primary clinical endpoint. Locoregional control was evaluated as a secondary endpoint since the vast majority of head and neck cancers recur either locally at the primary site or regionally in the neck; cause specific survival was also assessed. All curves were computed using the Kaplan-Meier method starting from the time of study entry. Curves for different subgroups were compared by the Cox-Mantel test. A chi-squared test was used to compare the clinical characteristics between M6P/IGF2R informative and excluded patients and informative patients with and without M6P/IGF2R loss of heterozygosity. A p-value < 0.05 was considered to be statistically significant.

Results

M6P/IGF2R *Loss of Heterozygosity Analysis*

The study population consisted of 116 patients enrolled in a phase III randomized clinical trial comparing radiation alone versus radiation plus concurrent chemotherapy for advanced head and neck cancer [31] plus an additional 23 patients who met all the entrance criteria but declined enrollment. A total of 52 tumors could not used in this investigation because either the tissue slides were unavailable or the DNA could not be PCR amplified. Of the remaining 87 patients, 56 (64%) were informative (i.e. polymorphic), and the tumors in 54% (30/56) of these patients had *M6P/IGF2R* loss of heterozygosity (Figure 1). The *M6P/IGF2R* informative patients and those not used in this study were insignificantly different from each other for the clinical characteristics of gender, age, race, smoking history, baseline hemoglobin, Karnofsky performance status, tumor resectability, treatment, tumor stage, nodal stage, overall staging and site of primary tumor (Table 1).

Clinical Outcome

The median follow-up for surviving patients enrolled on this trial [31] is now 76 months (range: 2 to 128 months). *M6P/IGF2R* loss of heterozygosity was associated with significant reductions in 5 year relapse-free survival {37% (95% CI: 20% to 54%) vs. 65% (95% CI: 46% to 84%); p = 0.05}, locoregional control {46% (95% CI: 28% to 64%) vs. 76% (95% CI: 58% to 94%); p = 0.03} and a non-significant reduction in cause-specific survival {43% (95% CI: 25% to 61%) vs. 69% (95% CI: 49% to 89%); p = 0.1}.

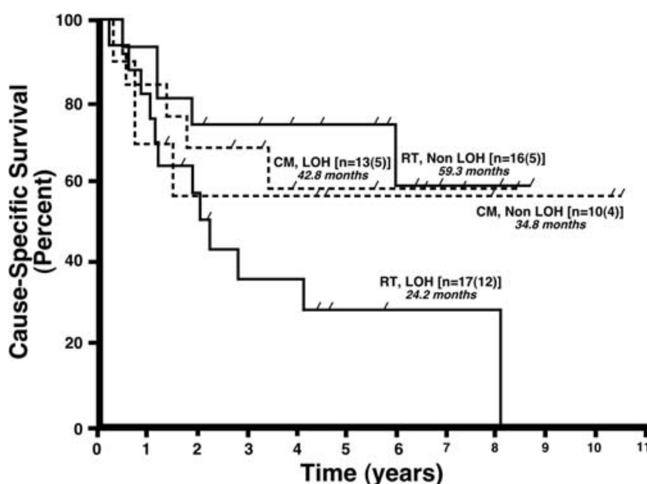

Figure 4
Kaplan-Meier estimates of cause specific survival in patients with head and neck cancer. Tumors treated with radiotherapy alone (solid lines) were either mutated (RT, LOH) or unmutated (RT, non LOH) at the *M6P/IGF2R* locus. Tumors treated with combined modality therapy (dashed lines) were also either mutated (CM, LOH) or unmutated (CM, non LOH) at the *M6P/IGF2R* locus. Patient number (n), patients that failed (parentheses), censored patients (angled bars), and median follow-up times are provided for each patient group. RT, radiotherapy; CM, combined modality therapy; LOH, loss of heterozygosity.

Since the clinical trial demonstrated a significant benefit for patients receiving radiotherapy and concurrent chemotherapy [31], outcome was also analyzed according to whether or not patients received chemotherapy (Figures 2,3,4). Patients in the radiotherapy (RT), loss of heterozygosity (LOH) group had significantly lower 5 year relapse-free survival {23% (95% CI: 2% to 44%) vs. 69% (95% CI: 46% to 92%); p = 0.02} (Figure 2), locoregional control {34% (95% CI: 11% to 57%) vs. 75% (95% CI: 54% to 98%); p = 0.03} (Figure 3) and cause specific survival {29% (95% CI: 5% to 53%) vs. 75% (95% CI: 54% to 96%); p = 0.02} (Figure 4) than those in the RT, non LOH group. These results indicate that *M6P/IGF2R* allelic loss results in poor patient outcome when RT alone is employed since all other measured clinical characteristics of the head and neck cancer patients were comparable to those in patients with a non-mutated *M6P/IGF2R* tumor suppressor gene (Table 1).

Patients in the RT, LOH group also fared worse than those in the combined modality (CM), LOH group, although the differences were not statistically significant in these smaller subgroups: 5 year relapse-free survival {23% (95% CI: 2% to 44%) vs. 54% (95% CI: 26% to 82%); p = 0.18}, locoregional control {34% (95% CI: 11% to 57%) vs. 61% (95% CI: 34% to 88%); p = 0.25} and cause specific survival {29% (95% CI: 5% to 53%) vs. 59% (95% CI: 30% to 88%); p = 0.12}. Patients in the CM, LOH group had 5 year relapse-free survival, locoregional control and cause specific survival that were statistically indistinguishable from those in the CM, non LOH group (p > 0.2). Combined modality therapy also did not provide any significant benefit over radiation alone for patients with a non-mutated tumor *M6P/IGF2R* (p > 0.2). Thus, patients with a non-mutated *M6P/IGF2R* who received RT alone had the same long-term outcome as the overall population of patients who received RT and concurrent chemotherapy [31]. This implies that *M6P/IGF2R* allelic loss may help to identify a group of head and neck cancer patients who can be adequately treated with RT alone without exposure to the added morbidity of combined modality therapy.

The development of second primaries was independent of the *M6P/IGF2R* mutation status in the primary tumor (p = 0.8). Three of the 30 (10%) patients with *M6P/IGF2R* loss of heterozygosity and two of the 26 (8%) patients without loss of heterozygosity at this locus developed second primaries.

Discussion

M6P/IGF2R loss of heterozygosity occurs frequently in human breast, liver and lung cancer [6,20–22], and the remaining allele of 30 to 50% of these tumors contains an intragenic loss-of-function point mutation in the ligand binding domains [35]. The *M6P/IGF2R* is also commonly mutated in gastrointestinal and endometrial malignancies because its coding sequence contains a poly-G region that is a mutational target in tumors with mismatch repair deficiencies and microsatellite instability [23,24]. Functional studies show that the introduction of an exogenous wild-type *M6P/IGF2R* into human colorectal cancer cells with a single inactivated allele significantly decreases growth rate and enhances apoptosis [26]. Conversely, loss of *M6P/IGF2R* expression promotes cancer cell growth by increasing intracellular signaling from both the insulin-like growth factor I receptor and the insulin receptors [36].

We demonstrate herein that loss of heterozygosity at the *M6P/IGF2R* locus in head and neck cancer is also associated with poor patient prognosis. Loss of heterozygosity in cancer can occur either because of chromosomal deletion or somatic recombination resulting in uniparental disomy [37]. Comparative genomic hybridization studies in head and neck cancer [10,38] demonstrate that 6q deletion frequency (> 50%) is similar to that which we observed at the *M6P/IGF2R* locus. This provides evidence that *M6P/IGF2R* loss of heterozygosity in head and neck

cancer is due primarily to either gene deletion or chromosomal loss rather than somatic recombination.

Since chromosomal deletion can affect more than one gene, *M6P/IGF2R* loss of heterozygosity alone does not rule out the possibility that other adjacent genes also have a tumor suppressor function in head and neck cancer. However, our previous finding, that both alleles of the *M6P/IGF2R* are mutated in greater than 50% of squamous cell carcinomas of the lung [22], supports our postulate that the *M6P/IGF2R* is a key head and neck cancer tumor suppressor gene at chromosome location 6q.

The clinical trial from which the tumor specimens used in this study were derived showed improvements in relapse-free survival, locoregional control, and overall survival for those patients randomized to combined modality therapy [31]. This investigation established that patients whose tumors had *M6P/IGF2R* loss of heterozygosity and were treated with radiotherapy alone had a significantly worse prognosis than their counterparts with a non-mutated allele. Conversely, patients with an intact *M6P/IGF2R* had a similar prognosis whether they received radiotherapy alone or combined modality treatment. Thus, our findings suggest that head and neck cancer patients with tumor *M6P/IGF2R* loss of heterozygosity would benefit most from combined modality treatment.

The mechanism by which *M6P/IGF2R* allelic loss in head and neck tumors increases the effectiveness of adjuvant chemotherapy is presently unknown, but it is clearly of clinical importance. A primary function of this receptor in placental mammals involves the degradation of extracellular IGF2. *M6P/IGF2R* allelic loss would therefore result in enhanced cellular bioavailability of this potent growth factor, thereby potentially increasing both cell proliferation and resistance to apoptosis [29]. The M6P/IGF2R also facilitates the activation of TGFβ, a potent cell growth inhibitor that is secreted in an inactive form (reviewed in [15,16]). *M6P/IGF2R* mutation is therefore one mechanism by which cancer cells can become refractory to TGFβ's mitoinhibitory effect.

We have previously shown that *M6P/IGF2R* mutation in squamous cell carcinoma of the lung is highly correlated with increased TGFβ concentrations in both the tumor and patient plasma [39]. Elevated TGFβ in the latter stages of tumor progression directly contributes to enhanced tumor angiogenesis, metastasis formation and a decreased host immune response (reviewed in [40]). Together these findings predict that tumors with a mutated *M6P/IGF2R* would be more resistant to therapy than those with an intact receptor. Moreover, if some chemotherapeutic agents are not only directly cytotoxic to cancer cells, but also reduce tumor production of growth factors, such as IGF2 and TGFβ, adjuvant chemotherapy would be more useful in treating tumors with a mutated *M6P/IGF2R*, as observed in this study.

M6P/IGF2R is normally imprinted in mice with only the maternal copy of the gene being expressed [41]. In contrast, both copies of the *M6P/IGF2R* are expressed in humans because genomic imprinting at this locus was lost in the primate lineage approximately 75 million years ago [42]. Importantly, restoration of biallelic *M6P/IGF2R* expression in mice results in a marked reduction in offspring weight late in embryonic development that persists into adulthood [43]. This demonstrates that *M6P/IGF2R* allelic loss or haploid insufficiency markedly enhances cell proliferation and/or survival during fetal development. Therefore, mutation of even a single allele of the *M6P/IGF2R* in human somatic cells is predicted to also promote cell growth.

Haploid insufficiency for tumor suppressor genes, such as *Nf2*, $p27^{Kip1}$, *p53*, *Ptch*, *Pten* and *TGFβ*, is known to promote tumor formation (reviewed in [44,45]). Yamada *et al.* [6] demonstrated that in patients chronically infected with hepatitis B and/or hepatitis C viruses, the *M6P/IGF2R* is mutated not only in hepatocellular carcinomas (HCCs), but also in the phenotypically normal hepatocytes adjacent to these tumors. Interestingly, only one *M6P/IGF2R* allele is inactivated in the adjacent cirrhotic tissue even when both alleles are mutated in the HCC. These findings are consistent with normal appearing, preneoplastic hepatocytes forming clonal masses in the liver because *M6P/IGF2R* haploid insufficiency affords them with a selective growth and/or survival advantage relative to normal hepatocytes [46].

The liver is not the only organ in which regions of normal appearing tissue have a clonal origin. Entire lobules and large ducts of normal breast tissue can be derived from a single progenitor cell [47], and LOH at various chromosomal locations is frequently detectable in morphologically normal lobules adjacent to breast tumors [48]. Lung tumors and head and neck tumors that develop in a localized region also often have a common clonal origin [3,4,49]. The high frequency of *M6P/IGF2R* loss of heterozygosity observed in the head and neck tumors in this study suggests that the phenomenon of "field cancerization", first described by Slaughter and his colleagues [2] in 1953, may in part result from the clonal proliferation of mucosal epithelial cells with *M6P/IGF2R* allelic loss.

Conclusions

The observations in this study raise the intriguing possibility that selection of head and neck cancer patients for concurrent chemotherapy can be based upon the

intratumoral mutational status of *M6P/IGF2R*. Furthermore, since *M6P/IGF2R* loss of heterozygosity in squamous cell carcinomas is highly correlated with an elevated plasma TGFβ level [39], patient prognosis may potentially be assessed by a simple blood test. This would be desirable since the morbidity of combined modality therapy is greater than that of radiotherapy alone [50,51].

Competing interests

None declared.

Authors' Contributions

TAJ conceived of the study, performed LOH determinations and drafted the manuscript. DMB provided patient samples and assisted in editing of manuscript. JKK, YO, H-SJ and XF identified *M6P/IGF2R* SNPs and performed LOH determinations. RTV confirmed tumor histology while RWC performed statistical analysis of the data. MSA assisted in experimental design and editing of manuscript. RLJ conceived of the study, participated in its design and coordination, and assisted in editing of manuscript. All authors read and approved the final manuscript.

List of Abbreviations

CIS, carcinoma *in situ*; HCC, hepatocellular carcinoma; IGF2, insulin-like growth factor 2; LOH, loss of heterozygosity; M6P/IGF2R, mannose 6-phosphate/insulin-like growth factor 2 receptor; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; TGFβ, transforming growth factor beta; UTR, untranslated region.

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