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CLINICAL INVESTIGATION **Head and Neck**

TUMOR EXPRESSION OF MAJOR VAULT PROTEIN IS AN ADVERSE PROGNOSTIC FACTOR FOR RADIOTHERAPY OUTCOME IN OROPHARYNGEAL CARCINOMA

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Purpose: Vaults are multi-subunit structures that may be involved in nucleo-cytoplasmic transport, with the major vault protein (MVP or lung resistance-related protein [LRP]) being the main component. The MVP gene is located on chromosome 16 close to the multidrug resistance-associated protein and protein kinase c-β genes. The role of MVP in cancer drug resistance has been demonstrated in various cell lines as well as in ovarian carcinomas and acute myeloid leukemia, but nothing is known about its possible role in radiation resistance. Our aim was to examine this in head-and-neck squamous cell carcinoma (HNSCC).

Methods and Materials: Archived biopsy material was obtained for 78 patients with squamous cell carcinoma of the oropharynx who received primary radiotherapy with curative intent. Immunohistochemistry was used to detect MVP expression. Locoregional failure and cancer-specific survival were estimated using cumulative incidence and Cox multivariate analyses.

Results: In a univariate and multivariate analysis, MVP expression was strongly associated with both locoregional failure and cancer-specific survival. After adjustment for disease site, stage, grade, anemia, smoking, alcohol, gender, and age, the estimated hazard ratio for high MVP (2/3) compared with low (0/1) was 4.98 (95% confidence interval, 2.17–11.42; $p = 0.0002$) for locoregional failure and 4.28 (95% confidence interval, 1.85–9.95; $p = 0.001$) for cancer-specific mortality.

Conclusion: These data are the first to show that MVP may be a useful prognostic marker associated with radiotherapy resistance in a subgroup of patients with HNSCC. © 2007 Elsevier Inc.

MVP, LRP, Radioresistance, HNSCC, Tongue, Tonsil.

INTRODUCTION

Vaults are barrel-shaped cytoplasmic ribonucleoprotein particles composed of multiple copies of three proteins. The mammalian vault complex is made up of major vault protein (MVP or LRP, M(r) 100,000), vault poly ADP-ribose polymerase (VPARP, M(r) 193,000), and telomerase-associated protein 1 (TEP-1 M(r) 240,000) that are associated with small 88- to 141-bp fragments of untranslated RNA (1–4). The major component of the vault complex is MVT, which constitutes more than 70% of the total mass. Vaults were first observed in clathrin-coated vesicles (5), and the first evidence that these structures may contribute to drug resistance was provided when lung resistance–related protein (LRP) was identified as human MVP (6). Although vaults are found in all human tissues, elevated levels of MVP are found in gut epithelium, lung epithelium, macrophages, and dendritic cells, which are all typically exposed to xenobiotics (5, 7, 8). This implies that vaults may have a role in the defense of such tissues against toxic insult. Consistent with this hypothesis, MVP has been found to be overexpressed in various multidrug-resistant cancer cell lines (9, 10), together with a range of clinical samples such as ovarian carcinomas

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and acute myeloid leukemia (11, 12). Although the majority of experimental and clinical investigations suggest that elevated expression at diagnosis is an independent adverse prognostic factor for response to chemotherapy for a variety of tumor types (12), not all studies support a role for MVP in chemoresistance (13).

The MVP gene is located on chromosome 16 close to the genes coding for multidrug resistance–associated protein and protein kinase c-β. Because the hollow barrel-shaped structure of the vault complex and its subcellular localization (14) indicate a function consistent with intracellular transport, it was postulated that vaults contributed to drug resistance by transporting drugs away from their intracellular targets and/or the sequestration of drugs (12). However, there are conflicting reports on the correlation and role of elevated MVP expression in drug resistance, and the exact mechanism remains to be defined (13, 15). Part of the problem is that many studies have only used univariate analysis, and it is clear that a multivariate approach is required to define more accurately any association of MVP with therapy resistance (12).

It is well known that radiation treatment can induce resistance to various cytotoxic drugs (16), although the mechanisms underlying this effect are complex (17). Furthermore, it is highly pertinent to the current study that recent work by Shimamoto *et al.* demonstrated that various DNA damaging agents, including ultraviolet irradiation, induce increased MVP transcription and protein levels (18). This implies that vaults may have a role in facilitating DNA repair processes, which is consistent with previous work showing that VPARP- and, to a lesser extent, TEP1-deficient mice have an increased incidence of carcinogen-induced colon tumors (19). It is well known that tumor-associated inappropriate activation of DNA repair mechanisms can greatly increase resistance to therapies that target DNA (20, 21).

These observations prompted us to investigate a potential association of MVP expression with outcome in a group of radiotherapy-treated oropharyngeal carcinoma patients. In a multivariate analysis of data from these patients, we show for the first time that elevated MVP expression is associated with a radiotherapy-resistant subset of patients with head and neck squamous cell carcinoma (HNSCC).

METHODS AND MATERIALS

Patient characteristics

This was a retrospective study performed during 1996 to 2001. The study involved patients with a histologically confirmed squamous cell carcinoma from two anatomic subsites of the head and neck, specifically the posterior third of tongue and the tonsil. The patients were identified from the Cancer Registry held at the Christie Hospital, with ethics approval being granted by the South Manchester Research Ethics Committee. The patient case notes were examined, and a database was generated with clinical information as outlined in Table 1. A total of 78 HNSCC patients were studied, comprising 37 patients with tonsillar squamous carcinomas and the remainder squamous carcinoma of the posterior tongue. Of the patients, 59 have died, and the median follow-up for the remaining 19 was 5.5 years (range, 1.5–8.0 years). Locoregional failure occurred in 41 patients, recorded at 6 weeks posttreatment, as either residual disease or recurrence after initial cure. The recorded locoregional failures occurred before any distant failure, as the first event. Six patients experienced distant metastases and 41 patients died from cancer-related causes. None of the patients developed a second primary site.

Treatment details

The patients all received radical radiotherapy to their primary site and, depending on the nodal status of their necks, also underwent a neck dissection. Megavoltage radiotherapy was delivered by means of a 4-MV linear accelerator in daily fractions five times per week for 3 weeks. The prescribed radiation dose at the time for posterior tongue tumors was 50 Gy using lateral parallel pair radiation in 16 fractions. Tonsillar tumors received ipsilateral therapy of 52.50 Gy in 16 fractions. None of this cohort of patients received chemotherapy.

Immunohistochemistry

The tissue blocks were received at the histology laboratories at the Paterson Institute, where slides were cut from these blocks and examined by a senior oral pathologist to confirm the presence of squamous cell carcinoma. Briefly, 4-μm sections were cut from formalin-fixed, paraffin wax–embedded pretreatment biopsy samples. These were dewaxed, and antigen retrieval was carried out by microwaving in 10 mmol/L citrate buffer (pH 6.0) for 25 min followed by blocking of the endogenous peroxidase by immersing the slides in 250 ml methanol containing 2.5 ml hydrogen peroxide solution for 30 min. Anti-MVP mouse monoclonal antibody (ab2376, Abcam) at 1:200 dilution in Tris-buffered saline (TBS) was applied to each slide and the slides incubated for 1 h at room temperature followed by 3×5 min washes with TBS. Biotinylated anti-mouse secondary antibody (DAKO EO433; Ely, United Kingdom) at 1:200 in TBS was applied for 30 min at room temperature, again followed by 3×5 min washes with TBS. Finally the sections were incubated with streptavidi-biotin complex (ABC reagent, DAKO 0377) at 1:500 for 30 min at room temperature, washed with TBS, and then stained with 3,3′-diaminobenzidine (DAKO K3465). After counterstaining with hematoxylin, the slides were dehydrated and mounted. Substitution of the primary antibody with the identical concentration of mouse IgG_1 (DAKO X0931) served as a negative control. Batch-to-batch variation was assessed by choosing two sections showing high and low MVP expression and running additional sections from these biopsy samples with each batch.

Analysis of scoring

A semi-quantitative system was used to estimate the percentage area of tumor cells that had been stained. The scoring system was as follows: 0 = no nuclear staining; 1 = $<$10% nuclear staining; 2 = 10% to 29% nuclear staining; and 3 = $\geq$30% nuclear staining. Scoring was blinded and was performed independently by 2 experienced oral pathologists (N.T., P.S.), with resolution of any conflicting scores (n = 16) being done by discussion and consensus.

Statistical analysis

Actuarial estimations of the relationship of tumor MVP expression with locoregional recurrence and cancer-specific death were estimated using the cumulative incidence method. The cumulative incidence curves were compared in univariate analysis using Gray's test (22). The distribution of MVP data were studied in relation to clinico-

Table 1. Patient characteristics

Characteristic	No. of patients	Tonsil	Tongue
Gender			
Male	57 (73%)	29	28
Female	21 (27%)	8	13
Age (y)			
Median (range)	59.7 (31.60–91.9)	57.8 (40.5–91.9)	62.4 (31.6–86.5)
Tobacco use			
Yes	59 (76%)	26	33
No	10 (13%)	5	5
Unknown	9 (11%)	6	3
Alcohol			
None	6	2	4
Low	25	12	13
Moderate	13	5	8
High	21	9	12
Unknown	13	9	4
Site			
Posterior tongue	41 (52%)		
Tonsil	37 (48%)		
Follow-up (y)	59/78 dead	25/37	34/41
Median (range)	5.49 (1.45–7.99)	5.57 (1.45–6.20)	4.95 (3.93–7.99)
Stage			
I	3	1	2
II	10	6	4
III	19	8	11
IV	46	22	24
Grade			
0	31	14	17
1	38	16	22
2/3	9	7	2
Anemia			
No	52	25	27
Yes	25	11	14
Unknown	1	1	0

pathologic variables subgroup (tonsil, tongue base), alcohol consumption, smoking history, age, gender, pretreatment anemia, stage, and histologic grade. Anemia was judged according to sex-adjusted thresholds of 13.0 for men and 11.5 for women. Smokers were divided into two groups: those who had previously smoked and those who had never smoked. Alcohol history was divided into four categories: those who did not consume alcohol ("none"), those whose intake was less than half the recommended weekly amount (RWA) ("low"), those whose intake was less than or equal to the RWA ("moderate"), and those whose intake was above the RWA ("high"). The distribution of patient characteristics according to high/moderate or low/absent MVP expression was analyzed using Chi-square tests. Multivariate analysis was performed with a Cox proportional hazards model for locoregional failure and cancer-specific mortality to determine whether any effect of MVP expression observed in univariate analysis retained significance after adjustment for the covariates. In 63 cases there was a complete set of data, with the covariates of site, stage, grade, anemia, smoking, alcohol, gender, age, and MVP expression.

RESULTS

MVP expression

Immunohistochemical analysis of MVP expression was performed on a total of 78 patients with a positive scoring system based on the presence of strong nuclear and cytoplasmic co-expression. Pure cytosol expression with negative nuclear staining could be extensive, but was always very weak or absent and was regarded as background staining. It is known that MVP localizes strongly at nuclear pores and cells judged to be positively stained showed chromogen in the nucleus and cytoplasm. Results were 29 negative and 49 positive, of which 8 showed high, 13 moderate, and 28 weak expression. Although there were 16 cases with conflicting scores, these scores were between 2 and 3, which does not affect the split used for the multivariate analysis. The extent of MVP staining was found to be highly reproducible on serial sections taken from the same biopsy sample. Examples of high and low classifications of MVP staining intensity with negative controls are shown in Fig. 1.

Association with outcome

Figure 2 illustrates the cumulative incidence curves showing the relationship between MVP expression and outcome for the whole group and the subgroups of posterior tongue and tonsil. It is clear that there is a strong association between elevated MVP expression and locoregional failure ($p = 0.0001$) for the whole group. Similarly, increased MVP expression was ad-

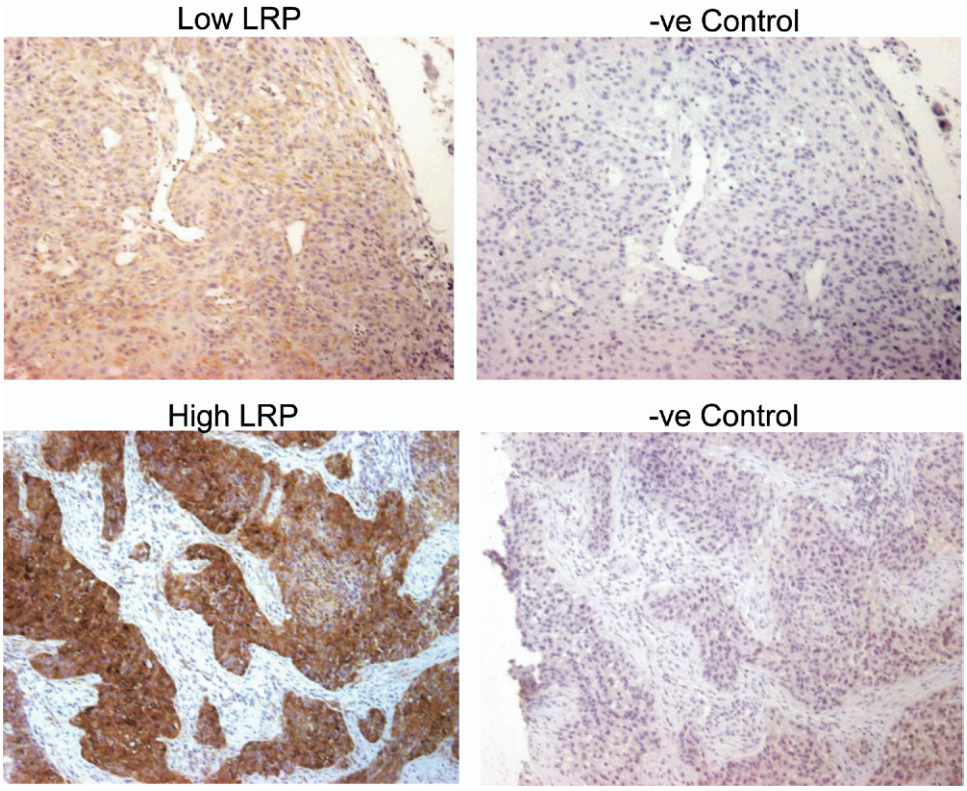

Fig. 1. Immunohistochemical analysis of major vault protein (MVP) expression in pretreatment tumor biopsy samples. Sections are examples of high and low expression plus negative controls. Expression of MVP was independently assessed by 2 oral pathologists and scored as follows: 0 = negative; 1 = low; 2 = moderate; 3 = high. LRP = lung resistance-related protein.

versely associated with cancer-specific death (Fig. 3) (p = 0.0007). On inspection of Fig. 2, the four scored groups appear to cluster into two, with patients who scored 0 and 1 having similar outcomes and with the same observed for patients who scored 2 and 3. On the basis of this observation, all further analyses were performed with just two MVP Groups 0/1 and 2/3. The estimated effects of MVP (2/3 vs. 0/1) appeared somewhat larger in the tongue cancer patients compared with the tonsil cancer patients (Figs. 2 and 3), with hazard ratios of 5.78 and 2.51 respectively for loco-regional failure and 5.09 and 2.43 for cancer-specific death. Formal tests for site–MVP interactions, however, were not statistically significant (p = 0.22 for loco-regional failure and p = 0.27 for cancer-specific survival).

Of the clinical variables studied, anemia was the only covariate that was significant in univariate analysis for both locoregional and cancer-specific mortality (p = 0.04 and p = 0.005 respectively).

Patient characteristics

To investigate whether any other factors or lifestyle practices might have influenced the results, we analyzed a range of patient characteristics. The data shown in Table 1 summarize the gender, age, smoking history, alcohol consumption, tumor site, and follow-up within the cohort of patients studied. The distribution of these patients according to MVP expression was tabulated and analyzed using the Chi-square test to investigate any association between MVP expression and each variable (Table 2). Crossmatching these characteristics with MVP expression indicates that the only significant association between any of these factors and the levels of MVP was with anemia (p = 0.02).

Multivariate analysis

For 63 patients there were complete sets of clinical data, allowing multivariate analyses to be carried out. The remaining 15 patients had incomplete information in terms of alcohol, smoking, and anemia. Site, disease stage, grade, gender, age, and MVP expression were also entered into the Cox proportional hazard model for locoregional failure and cancer-specific survival (Tables 3–5). After adjustment for all these covariates, MVP remained a highly significant independent prognostic factor for both outcomes. After adjustment for all the clinical factors mentioned above, the estimated hazard ratio for high MVP (2/3) compared with low (0/1) was 4.98 (95% confidence interval [CI], 2.17–11.42, p = 0.0002) for locoregional failure and 4.28 (95% CI, 1.85–9.95, p = 0.001) for cancer-specific mortality.

The aim of the multivariate analyses was to see whether the MVP effect observed in univariate analysis persisted after adjustment for a set of covariates thought to be important in HNSCC. The focus being on the MVP effect estimates with and without adjustment, no great attention being given to the estimates and significance of the other

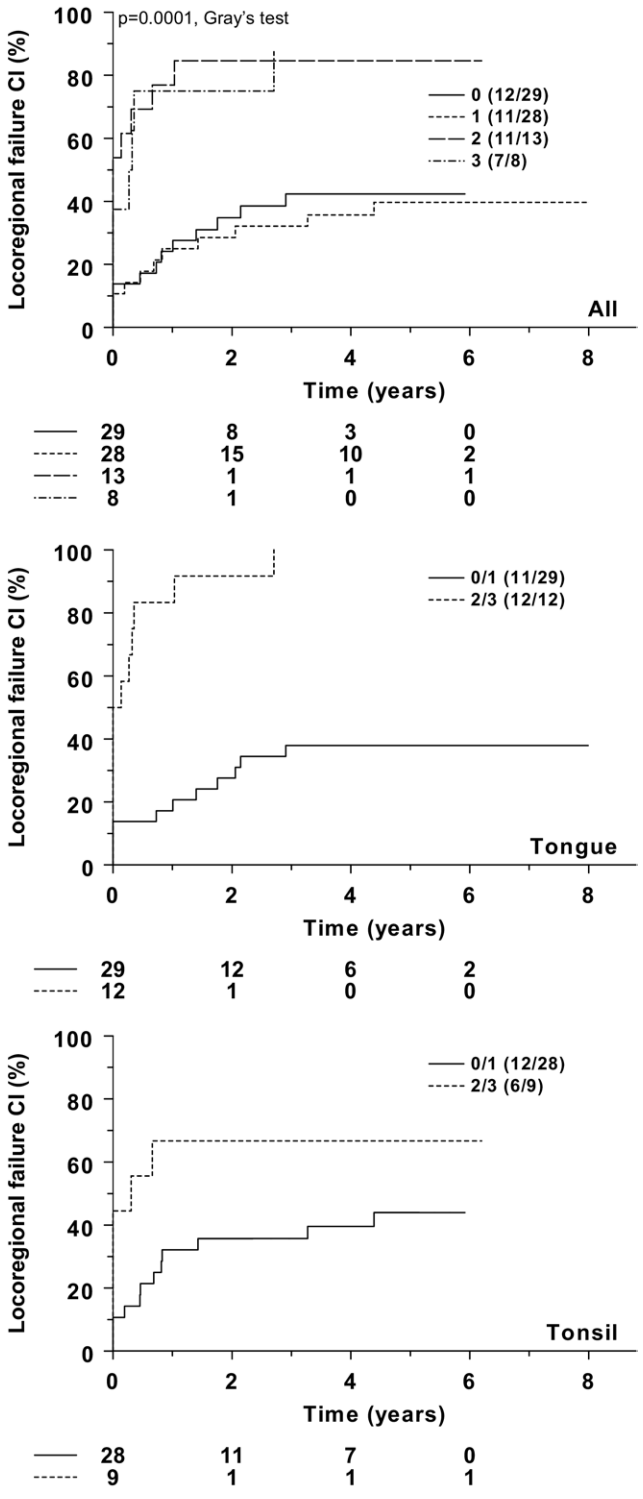

Fig. 2. Cumulative incidence (CI) curves showing the relationship between tumor major vault protein (MVP) expression and locoregional failure after radiation therapy in 78 patients with head-and-neck squamous cell carcinoma (HNSCC). These data indicate the strong association between MVP expression and locoregional failure in moderate to high MVP expression in HNSCC of the posterior tongue.

terms in the models because of the modest number of events.

After an additional set of stepwise Cox regression analyses, for both outcomes the only covariate, other than MVP, that was significant in univariate analysis was anemia ($p = 0.04$ and $p = 0.005$ for locoregional failure and cancer-specific mortality, respectively). None of the remaining covariates approached statistical significance after adjust-

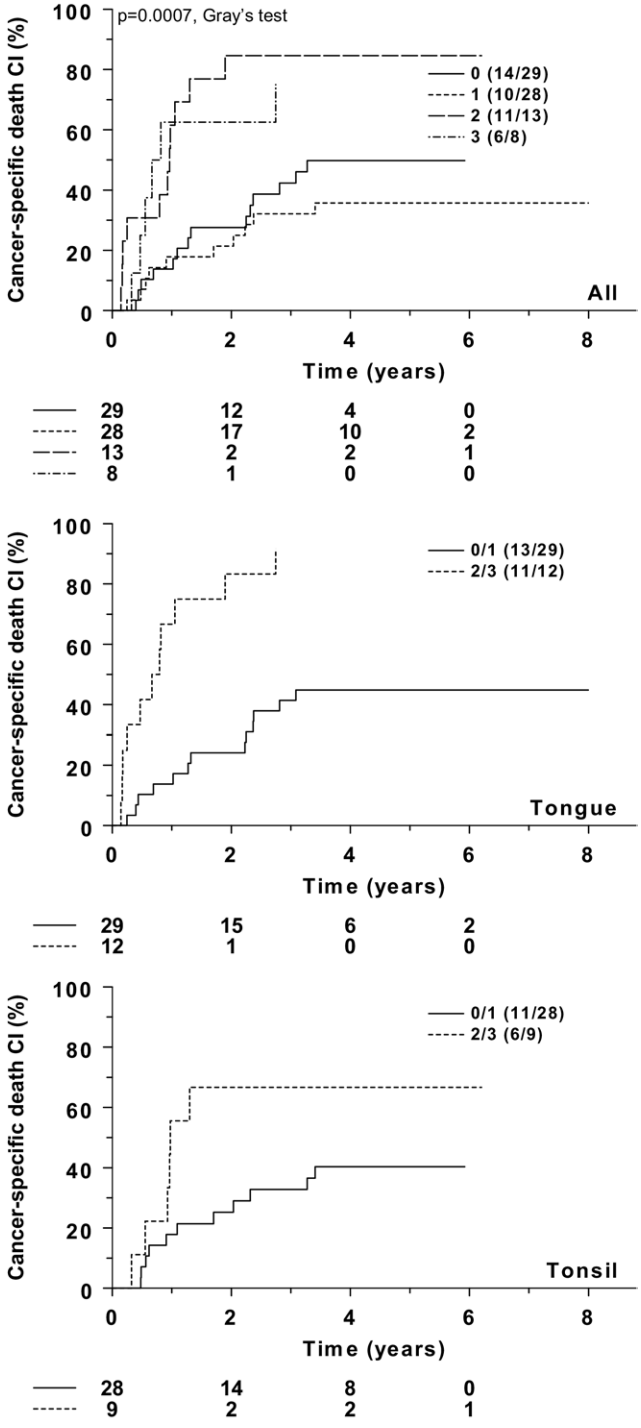

Fig. 3. Cumulative incidence (CI) curves showing the relationship between tumor major vault protein (MVP) expression and cancer-specific mortality after radiotherapy in 78 patients with head-and-neck squamous cell carcinoma (HNSCC). The data show the strong association between MVP and cancer-specific mortality in oropharyngeal carcinomas.

Table 2. Association of patient characteristics with major vault protein (MVP) expression (scored 2 or 3) in 21/78 patients

	High MVP expression	
Characteristic	*n*	*p* value
Site		
Tonsil	9/37	
Tongue	12/41	0.62
Alcohol history		
None	1/6	
Low	6/25	
Moderate	4/13	
High	6/21	0.91
Smoking history		
Negative	1/10	
Positive	16/59	0.24
Age		
<60 y	10/40	
≥60 y	11/38	0.69
Anemia		
No	10/52	
Yes	11/25	0.020
Stage		
I	0/13	
II	3/10	
III	3/19	
IV	15/46	0.37

ment for anemia, with the smallest p value being 0.15. However, MVP remained highly significant after adjustment for anemia with an estimated hazard ratio of 4.16 (95% CI, 1.98–8.77, $p = 0.0003$) for locoregional failure and 3.30 (95% CI, 1.55–6.99, $p = 0.003$) for cancer-specific mortality.

DISCUSSION

Head and neck cancer describes a heterogeneous group of patients among whom identically staged tumors can exhibit widely differing response to therapy despite receiving identical treatment regimens. The ability to predict treatment outcomes, particularly with regard to response to radiotherapy, would provide the means of individualizing patient management—for example, the choice of primary treatment modality or whether adjuvant treatment would be required. Such individualization needs to be determined not only on the basis of conventional clinical parameters but also on biologic risk factors (23). Our analysis indicates that

Table 4. Multivariate Cox model for locoregional failure (32/63)

Variable	HR	95% CI	*p* value
Site			0.81
Tonsil (reference)			
Tongue	1.10	0.51–2.37	
Stage			0.73
I /II (reference)			
III/IV	0.81	0.25–2.61	
Grade			0.07
0 (reference)			
1–3	0.45	0.19–1.07	
Anemia			0.23
No (reference)			
Yes	1.79	0.71–4.51	
Smoking			0.95
Never (reference)			
Ever	0.96	0.28–3.26	
Alcohol			0.26
None/low (reference)			
Mod/high	1.68	0.68–4.15	
Gender			0.64
Male (reference)			
Female	1.26	0.48–3.28	
Age			0.76
<60 (reference)			
≥60	1.14	0.48–2.72	
MVP			0.0002
0/1 (reference)			
2/3	4.98	2.17–11.42	

Abbreviations: CI = confidence interval; HR = hazard ratio; MVP = major vault protein.

Table 3. Univariate Cox models for all 78 cases and the subset of 63 cases with complete data used in the multivariate analyses

Outcome	Events/*n*	Variable	HR	95% CI	*p* value
Locoregional failure	41/78	MVP			<0.0001
		0/1 (reference)			
		2/3	4.14	2.16 to 7.94	
Locoregional failure	32/63	MVP			0.0002
		0/1 (reference)			
		2/3	4.28	2.06 to 8.89	
Cancer death	41/78	MVP			0.0002
		0/1 (reference)			
		2/3	3.56	1.88 to 6.73	
Cancer death	30/63	MVP			0.002
		0/1 (reference)			
		2/3	3.39	1.62 to 7.13	

Abbreviations: CI = confidence interval; HR = hazard ratio; MVP = major vault protein.

Table 5. Multivariate Cox model for cancer-specific mortality (30/63)

Variable	HR	95% CI	*p* value
Site			0.03
Tonsil (reference)			
Tongue	2.67	1.10–6.50	
Stage			0.62
I/II(reference)			
III/IV	1.37	0.38–4.95	
Grade			0.07
0 (reference)			
1–3	0.45	0.19–1.07	
Anemia			0.02
No (reference)			
Yes	3.29	1.19–9.13	
Smoking			0.46
Never (reference)			
Ever	1.65	0.41–6.60	
Alcohol			0.58
None/low (reference)			
Moderate/high	0.77	0.30–1.94	
Gender			0.89
Male (reference)			
Female	1.07	0.41–2.82	
Age			0.27
<60 (reference)			
≥60	0.58	0.22–1.54	
MVP			0.001
0/1 (reference)			
2/3	4.28	1.85–9.95	

Abbreviations: CI = confidence interval; HR = hazard ratio; MVP = major vault protein.

MVP expression levels may provide the means of identifying those patients most at risk for treatment failure, although the underlying mechanisms behind this observation are not understood.

It is still unclear how vaults mediate drug resistance in cancer cells, and various theories have been suggested. The majority of vaults are present in the cytoplasm, although a subset localize to the nuclear membrane at or near the nuclear pore complexes (14). By virtue of this location, these structures may act to bind drugs or drug-binding proteins, and either sequester or transport them to cellular locations that are remote from the cellular drug target (12, 14). Alternatively, the vaults may act to regulate the function of other proteins that are indirectly related to drug resistance. For example the tumor suppressor phosphatase and tensin homolog deleted on chromosome 10 (PTEN) protein has been shown to interact with MVP in Hela cells (24). PTEN dephosphorylates phosphatidyl inositol-3,4,5-P_3, to negatively regulate the phosphoinositide 3-kinase pathway and thus regulate cell growth. In addition, PTEN is frequently found mutated in a variety of tumors (25), where downregulation of its activity and/or transient translocation to the cell membrane induces uncontrolled growth. Interestingly, it has been shown that interaction with MVP is necessary for transport of PTEN from the cytoplasm to the nucleus, which peaks at the G0 to G1 stage of the cell cycle and declines during the S phase (26); yet the function of this cycle-dependent PTEN nuclear-cytoplasmic translocation remains unclear. A plausible explanation may be found in recent work that showed that nuclear PTEN decreased the level and nuclear localization of cyclin D1, resulting in cell cycle arrest, whereas cytoplasmic PTEN was required for apoptosis (27). High levels of MVP could thus promote the nuclear transport of PTEN from the cytoplasm, which may facilitate delayed entry into the cell cycle and also suppress apoptosis. Because cytotoxic drugs and radiation are most effective against rapidly dividing cells, this could facilitate increased resistance to these agents by prolonging the cell cycle to allow sufficient time for DNA repair mechanisms to operate (28). Clearly this hypothesis would depend on the PTEN present in any particular tumor being of the wild type, and would indicate that analysis of the mutation status and nuclear levels of this protein in HNSCC tumors may prove informative.

Evidence in support of this hypothesis is provided by the observation that increased MVP expression has been shown to be associated with the drug-induced cytostatic stress response of malignant cell (29). Indeed recent work has shown that MVP interacts with constitutively photomorphogenic protein 1 (COP1), which is a RING finger ubiquitin ligase with substrates including c-Jun and p53 (30). It was demonstrated that ultraviolet radiation caused an increase in tyrosine phosphorylation of MVP, which caused it to dissociate from COP1, which suppresses the negative regulatory effects of this protein on c-Jun transcription. Thus, MVP and COP1 appear to act in concert to suppress c-Jun–mediated transcription of activator protein–1 (AP-1) under stressed conditions. These observations clearly support the contention that high levels of MVP may facilitate an enhanced cytostatic response to drug or radiation treatment in some types of cancer, which may provide the necessary time for repair of potentially lethal damage.

It is now accepted that many human cancers may have viral etiologies (31), and subsets of HNSCC have been linked to infection with high-risk human papilloma virus (32). Indeed the differences suggested by our subsite analyses, although not retained on a multivariate analysis, might be in part explained by their viral etiology.

It is significant that constitutive expression of the human T-cell leukemia virus type 1 (HTLV1) Tax oncoprotein induces expression of MVP and associated drug resistance in an adult T-cell leukemia cell line. Furthermore there are some known similarities in the mode of action of the HPV16 E6 and HTLV1 Tax oncoprotein (33). Yet HPV-positive HNSCCs generally have a better prognosis than HPV-negative tumors, which would not support HPV-mediated induction of MVP expression and associated therapy resistance. However, it has been shown that HPV-positive HNSCCs, which also have a p53 mutation, carry an extremely poor prognosis (34, 35). Indeed we have shown that expression of HPV16 E6 in mutant p53–expressing, human C33A cervical carcinoma xenografts, confers extreme radiation resistance (20). These data clearly indicate that viral oncoproteins, such

as HPV E6, can synergize with additional events, exemplified by p53 mutation, to exacerbate the disease process.

In summary, the data reported here suggest that MVP might be a useful prognostic marker for patients with HNSCC, with suggestion of a different effect in tumors of the posterior tongue. A larger study combined with additional work on p53 mutation status and nuclear PTEN levels will provide additional information necessary to confirm our findings and further define the underlying mechanism behind these effects.

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