Published in final edited form as:

Clin Cancer Res. 2012 January 1; 18(1): 301–307. doi:10.1158/1078-0432.CCR-11-2295.

Prognostic significance of plasma osteopontin in patients with loco-regionally advanced head and neck squamous cell carcinoma treated on TROG 02.02 Phase III trial

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Abstract

Background—High plasma osteopontin (OPN) levels have been reported to be an adverse prognostic factor in HNSCC, correlate with tumor hypoxia and be predictive of benefit from hypoxia targeted therapy. We sought to confirm the prognostic and predictive significance of OPN in patients treated on a large international trial.

Methods—Patients with Stage III/IV HNSCC were randomized to receive definitive radiotherapy concurrently with cisplatin or cisplatin plus the hypoxic cell cytotoxin, tirapazamine (TPZ). Eligibility criteria for this prospective substudy included plasma sample availability for OPN assay by ELISA and absence of major radiation therapy deviations (N=578). OPN concentrations were analysed for overall survival (OS) and time to locoregional failure (TTLRF), adjusting for known prognostic factors. Additional analysis was performed in patients with available tumor p16^{INK4A} staining status.

Results—The median OPN level was 544ng/mL (range: 7–2640). High OPN levels were not associated with worse OS (relative HR, 1.05 for highest tertile) or TTLRF (relative HR 0.90 for highest tertile). There was no interaction between OPN and treatment arm for OS or TTLRF (p=0.94 for OS; p=0.85 for TTLRF). For the highest tertile the 2-year OS was 66% on control arm and 67% on TPZ arm (HR=1.11, p=0.67). Similarly for p16^{INK4A} negative patients in the highest tertile, the 2-year OS was 61% on control arm and 63% on TPZ arm (HR=1.05, p=0.86).

Interpretation—We found no evidence that high plasma OPN levels were associated with an adverse prognosis in HNSCC, or were predictive of benefit with hypoxia targeting therapy.

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Conflicts of interest

There are no conflicts of interest to declare.

Keywords

“Osteopontin”; “Head and neck squamous cell carcinoma”; “prognostic”; “predictive”; “biomarker”

INTRODUCTION

Tumor hypoxia is associated with an adverse prognosis in head and neck squamous cell carcinoma (HNSCC).(1, 2) However, trials testing interventions targeting hypoxia have largely been done in unselected patients and results are generally disappointing. Furthermore, the field has been hampered by the lack of a validated, non-invasive test for hypoxia that can easily be performed. An attractive means of identifying tumor hypoxia is by the use of plasma endogenous markers.

Plasma osteopontin (OPN) has been reported to be a putative marker of tumor hypoxia in HNSCCs.(3, 4) OPN, a secretory phosphoglycoprotein, is a member of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family.(5) Elevated expression of OPN has been associated with advanced stage and poor outcome in multiple cancers including HNSCC, breast cancer and colorectal cancer(6). OPN secretion also occurs in response to inflammation, ischemic heart disease, renal disease and bone remodelling.(7–11)

OPN expression is inversely correlated with the expression of the Von Hippel Lindau (VHL) protein.(3) VHL protein modulates the expression of hypoxia induced factor-1 α (HIF-1 α) which regulates adaptive responses to hypoxia, as well as tumor metastasis and progression. Le and colleagues demonstrated an inverse relationship between OPN levels and tumoral oxygenation ($p=0.003$, $r=-0.42$), with multivariate analysis confirming that high OPN levels were associated with worse survival ($p=0.02$).(3) Further evidence for the potential of OPN to identify hypoxic tumors was provided by the DAHANCA 5 study, a large international study that demonstrated improved locoregional control for patients with locally advanced HNSCC treated with radiotherapy and the hypoxic sensitiser, nimorazole.(12) Patients with high OPN levels who received radiotherapy alone had worse outcome compared to those allocated nimorazole ($p=0.01$ for locoregional control and $p=0.0004$ for disease-specific survival).(4) For patients who did not have high OPN plasma concentrations, outcome according to treatment arm did not differ. For patients on the control arm, high OPN levels were associated with a worse prognosis compared to low OPN levels. Thus, high OPN levels were thought to reflect tumor hypoxia which could be counteracted by the use of hypoxia modulating therapy.

Based on this data, an *a priori* decision was made to examine the utility of OPN as a prognostic and predictive marker, in an international phase III trial that tested the benefit of adding tirapazamine (TPZ), a hypoxic cell cytotoxin, to cisplatin and radiotherapy for the treatment of locally advanced HNSCC.(13) Primary findings of the study demonstrated no overall survival benefit for the addition of TPZ to standard treatment but highlighted the critical importance of the quality of radiation therapy delivery.(14)

As human papillomavirus (HPV) status is a recognised favourable prognostic factor for patients with oropharyngeal squamous cell carcinomas, additional analysis was performed to investigate the relationship between OPN levels and p16^{INK4A} immunohistochemistry (IHC), a surrogate marker for HPV positivity status.(15) We reported in the HPV substudy of the TROG 02. 02 trial a non-significant trend for benefit from TPZ in p16^{INK4A} negative patients, with an improved time to locoregional failure. We hypothesized that this may be the subgroup where hypoxia confers an adverse prognosis. Here we evaluated whether

circulating OPN levels could help to further stratify patients in the p16^{INK4A} (HPV) negative group, and thus assist in selection of the patients for treatment with TPZ.

MATERIALS AND METHODS

Trial design

Details of the trial protocol have been previously described.(13) Briefly, the trial was performed between September 2002 and April 2005 in 82 international centres, and randomly assigned 861 patients with locoregionally advanced (Stage III–IV) HNSCC to definitive treatment with cisplatin (CIS) based chemoradiotherapy with or without TPZ (TPZ/CIS). Patients were assigned treatment centrally and were stratified according to disease stage (III v IV), primary site (oropharynx/larynx v hypopharynx/oral cavity), and haemoglobin level (135 g/L for men and 125 g/L for women). Patients received 70Gy of radiotherapy over seven weeks, with CIS 100mg/m² weeks 1, 4 and 7 for the CIS arm or CIS 75mg/m² with TPZ 290mg/m²/d on day 1 of weeks 1, 4, and 7 plus TPZ alone 160mg/m²/d on days 1, 3, and 5 the other weeks for the TPZ/CIS arm. Approval from participating institutional ethics committees was obtained and all patients provided informed consent. The primary endpoint for the trial was overall survival adjusted for major prognostic factors.

ELISA measurements of OPN level

The trial protocol stipulated instructions for uniform sample collection, transportation and storage. Patient plasma samples were prospectively collected into EDTA tubes, were processed within 30minutes of collection, transported at minus 20°C and stored at minus 80°C until delivered to the central laboratory (Stanford). When samples were received, plasma was aliquoted and stored at minus 80°C until analysis. As a quality assurance measure, to distinguish serum from plasma, fibrinogen levels were assessed in specimens with extremely low OPN values. Samples with consistently low fibrinogen levels were excluded from analysis, being indicative of serum rather than plasma. The commercially available enzyme-linked immunosorbent assay (ELISA) Human Osteopontin TiterZyme Eia System (IBL, Japan) was used to measure OPN levels. All samples were run in duplicates or triplicates. Plasma samples from two non-cancer patients were used to control for inter-assay variability.

P16^{INK4A} immunohistochemistry

P16^{INK4A} immunohistochemistry was used to define HPV positive patients. Methods have previously been reported in a substudy of the TROG 02. 02 trial(15). p16^{INK4A} IHC was performed using the mouse monoclonal anti-p16 (Lab Vision/NeoMarkers, Fremont, CA) on formalin-fixed paraffin embedded tissue sections. Intensity of p16^{INK4A} staining was determined by a pathologist, with absent or weak staining indicating a negative p16^{INK4A} status.

Statistical Analysis

Based on the DAHANCA 5 OPN substudy, (4) OPN concentrations were divided according to tertile and median values. Two-way frequency tables were analysed by standard chi-square tests. The Kaplan-Meier method was used to estimate overall survival (OS) and time to locoregional failure (TTLRF). OS and TTLRF were measured from the end of radiotherapy, because of the radiation therapy delivery deviation eligibility criterion. It was decided *a priori* that the analysis of the main objectives would be conducted adjusting for previously identified prognostic factors (primary site, T category, N category, ECOG performance status, and hemoglobin).(13) Groups were compared with respect to OS and TTLRF, using the logrank test and the Cox proportional hazards model, the latter being used

for the adjusted analyses. All p-values are two-sided. Hazard ratios (HR) for arm refer to TPZ/CIS:CIS. Analysis was undertaken using the R statistical package.(16)

RESULTS

Of 853 evaluable patients, 158 patients were excluded due to the following reasons; 87 for major deviations of radiation therapy delivery, 33 for non-evaluable radiotherapy plans, ten for not receiving treatment as planned, 24 for receiving less than 60Gy of radiotherapy and four patients for having progressed prior to treatment completion. Of the remaining 695 evaluable patients, an additional 117 patients were excluded; 43 for lack of a plasma sample and 74 patients for samples with low fibrinogen levels. Thus a total of 578 patients had plasma samples available for OPN analysis (Figure 1a). All patients analysed have had a minimum of two years follow up or have been followed up until the time of death.

A separate analysis was done for the p16^{INK4A} negative group (Figure 1b). For the purpose of this analysis the p16^{INK4A} negative group included only oropharyngeal patients who had been tested to be p16^{INK4A} negative, plus all non-oropharyngeal patients, giving a total of 335 patients analysed in this group. An additional analysis was also performed for 75 p16^{INK4A} negative oropharyngeal patients only.

The median OPN concentration was 544 ng/mL (range 7–2640 ng/mL). OPN concentrations were divided into tertiles according to high (> 711 ng/mL), middle (407–710 ng/mL) and low (< 407 ng/mL) concentrations. Table 1 demonstrates the patient and tumor characteristics according to OPN levels by tertile. Patients who had OPN concentrations in the highest tertile were more likely to be of an older age, to be male, have a higher T-stage and have a low haemoglobin level

Initial unadjusted analysis demonstrated no significant differences in OS between tertiles, although patients in the highest tertile demonstrated a slightly lower 2-year OS of 64% compared to 71% for both low and middle tertiles (relative HR are 1:1.00:1.27 for low, middle and high tertiles, p=0.29). When analysis was performed adjusting for known prognostic factors, as was the primary endpoint of our study (Figure 2), there were no differences between groups with 2-year OS rates of 71%, 72% and 70% for the low, middle and high tertiles (relative HR is 0.98 for middle tertile and 1.03 for highest tertile, p=0.95). TTLRF did not significantly differ according to OPN concentrations (relative HR 0.93 for middle tertile and 0.91 for highest tertile, p=0.91). Similarly, when analysis was restricted to the control arm, there were no significant differences in outcome according to OPN concentrations (data not shown). When OS and TTLRF were assessed within OPN tertiles according to treatment arm, there were no significant differences between treatment arms (Figure 3). For the highest tertile, the adjusted 2-year OS was 66% on CIS and 67% on TPZ/CIS (HR=1.11, 95% CI 0.69 to 1.77, p=0.67). There was no interaction between OPN and treatment arm for OS (p=0.93) or TTLRF (p=0.87). Similar negative results were observed in analyses looking at OPN as a dichotomous variable, split at the median, and at OPN as a continuous variable (data not shown).

As there was a suggestion in this trial that there may be better locoregional control on the TPZ/CIS arm in the p16^{INK4A} negative group, (15) we sought to determine whether OPN levels were prognostic or predictive in the p16^{INK4A} negative group. We found more p16^{INK4A} negative tumors in the highest OPN tertile, though the difference was not significant (p=0.21). There were no differences in OS among OPN tertiles for p16^{INK4A} negative patients (p=0.80). For the highest OPN tertile of the p16^{INK4A} negative patients, there was no difference in the OS between treatment arms (2-year OS 61% for the control and 63% for the study arm, HR=1.05, p=0.86). TTLRF in the highest OPN tertile of the

p16^{INK4A} negative group, also did not differ between treatment arms (HR=0.85, p=0.64). A separate analysis of only p16^{INK4A} negative oropharyngeal patients found similar negative results (data not shown).

DISCUSSION

This is the largest phase III study prospectively investigating the prognostic and predictive significance of OPN as a putative hypoxia marker in locoregionally advanced head and neck cancer. We could find no evidence of difference in outcome based on OPN levels in the whole population or in the control arm. Furthermore, we did not find any evidence that high OPN levels were predictive of benefit from treatment with the hypoxic cell cytotoxin, tirapazamine.

Hence, we were unable to confirm the results of a recent meta-analysis that reported an adverse prognosis in a variety of tumors (including HNSCC) with high OPN levels, or of previous studies showing that high OPN levels were predictive of benefit from the addition of agents targeting hypoxia in patients with HNSCC.(3, 4, 6)

The meta-analysis did not adjust for known prognostic variables with which OPN levels correlated. Also, some previous HNSCC reports need to be interpreted with caution due to the small sample size.(3, 17) However, the DAHANCA 5 trial which evaluated the treatment benefit of adding nimorazole, a hypoxic cell sensitizer, to radiotherapy was a large study of 320 patients.(12) The reasons for the discordant findings between the DAHANCA 5 study and our results are not immediately apparent, but there are some notable differences. The control arm in the DAHANCA trial was radiation alone and it is plausible that hypoxia has a greater impact on outcome in this setting compared to high dose chemoradiation.(13) In the DAHANCA trial, samples had been stored prior to analysis for up to 19 years, while our assays were performed soon after completion of accrual. The tertiles reported in the DAHANCA 5 study were upper (167–1382 ng/mL), middle (69–166 ng/mL) and lowest (0–68 ng/mL). Our OPN concentrations were much higher; high (711–2640 ng/mL), middle (407–710 ng/mL) and lowest (7–406 ng/mL). This highlights a significant decay in OPN levels over time, but it is not known whether this could impact on the reliability and interpretation of the results. In addition, our trial had extensive radiotherapy quality assurance and radiation therapy delivery deviations were predicted to lead to poor tumor control.(14) These cases had a significantly poorer prognosis and were excluded from this substudy. Information about such radiation therapy delivery deviations in the DAHANCA 5 trial is not available, nor is it possible to know how this may have impacted on the results. Our follow up is shorter than in the DAHANCA 5 study, though our patients had a minimum of 2-years of follow up. While the different mechanisms of action of the two hypoxia targeting therapies and the overall negative results of the TROG 02.02 trial may impact on whether high OPN levels are predictive of benefit from hypoxia targeting therapies, they do not explain the discordant findings about high OPN levels as an adverse prognostic factor in the control arm.

We have previously reported a non-significant trend favouring TPZ use in p16^{INK4A} negative patients, (15) with one possible explanation being that this is the group where hypoxia is particularly important as an adverse prognostic factor.(18) The improved outcome for p16^{INK4A} negative patients with hypoxia targeting therapy has also been reported by the DAHANCA group.(19) Though we found that p16^{INK4A} negative patients were more likely to have high circulating OPN levels, supporting the concept that OPN may identify patients with more aggressive tumor biology, this trend was not significant. Moreover, we did not find a difference in outcomes between those treated with or without TPZ in the entire p16^{INK4A} negative population or when analysed by within OPN tertiles.

The measurement of plasma OPN is problematic. There are several commercial antibodies that recognize OPN at different epitopes with different stringencies. The sensitivity and specificity of different OPN ELISA systems are highly dependent on the type of antibody used, washing stringency and the antigen recognized (total versus cleaved OPN). Two studies have found that different OPN ELISA systems reported different absolute values for the same samples, though the correlation coefficient was reasonably high.(20),(21) Other known contributors to circulating OPN measurement variability are the type of blood samples evaluated (serum versus plasma), the duration of sample storage and the number of freeze and thaw cycles. Secreted OPN can undergo post-translational modification, extracellular proteolytic cleavage by thrombin or metalloproteases, or be sequestered in protein complexes such as with the complement factor H.(22) These processes are highly variable for the different type of blood sample collected including serum, citrated plasma, EDTA plasma, or heparinized plasma. Therefore, to standardize OPN measurements in the study we collected only EDTA samples and excluded those that had low fibrinogen levels which may represent accidentally collected serum. The tested samples were only freeze-thawed once. To avoid the variability between different ELISA systems, we used the same OPN ELISA assay that was originally published in the initial study by Le and colleagues, (3) though now marketed under another name. The ELISA system used detected only full length OPN and measurements were performed centrally with one operator and one antibody lot to ensure consistency of results. The coefficient of variation (CV) of intra-assay precision was within ten percent and inter-assay precision was less than fifteen percent, which is consistent with most commercial ELISA systems. These safeguards ensure that the results are not influenced by sample collection issues or due to chance alone.

In conclusion, we were not able to confirm previous reports that support plasma OPN as a prognostic marker in head and neck cancer, nor did we find any evidence that it predicted benefit from the addition of a therapy targeting hypoxia. While therapeutic approaches targeting hypoxia require better methods to identify patients with significant tumor hypoxia e.g., hypoxic PET imaging, (27) our results suggest that further investigation of plasma osteopontin as a hypoxia marker is unlikely to be productive.

Acknowledgments

Nil.

GRANT SUPPORT

The research was supported by 1 R01 CA118582 and NHMRC project grant, P01-CA67166.

Dr Annette Lim was supported by the Spotlight Charitable Foundation Fellowship in Head & Neck Oncology and the Melbourne University Australian Postgraduate Award.

Financial support

1 R01 CA118582, NHMRC project grant, P01- CA67166, the Spotlight Charitable Foundation Fellowship in Head & Neck Oncology and, the Melbourne University Australian Postgraduate Award.

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Statement on Translational Relevance

Hypoxia is an adverse factor associated with poor outcome and treatment resistance in head and neck cancers (HNC). There is a need to identify biomarkers of tumor hypoxia that could assist in selection of patients for hypoxia targeted therapy. Plasma osteopontin (OPN) has been reported to be a putative marker of hypoxia in HNC and to be predictive of response to a hypoxic sensitiser. Here, we present the largest body of unbiased evidence from a phase III international prospective study investigating the role of OPN in HNC and the only study assessing its role in the HPV (p16INK4A) negative population. We found no evidence that OPN was a predictive or prognostic marker in unselected patients or in the p16INK4A negative group. This suggests further investigation of OPN as a hypoxic marker in HNC is unlikely to be productive and highlights this as an area of ongoing need.

Figure 1. a)

Figure 1. b)

Figure 1. Patient flow charts

a. Whole population

b. p16^{INK4A} negative population

Figure 2. Primary outcomes by osteopontin tertile, adjusting for prognostic factors (Kaplan-Meier curves). Two-year overall survival rates and relative hazard rates reflect the order of the legends

a. Overall survival

b. Time to locoregional failure

3a) OPN lower tertile: OS and TTLRF

3b) OPN middle tertile: OS and TTLRF

3c) OPN upper tertile: OS and TTLRF

Figure 3. Primary outcome by treatment arm according to OPN tertiles, adjusting for prognostic factors (Kaplan-Meier curves). Two-year overall survival rates and relative hazard rates reflect the order of the legends

- OPN lowest tertile: overall survival and time to locoregional failure
- OPN middle tertile: overall survival and time to locoregional failure
- OPN highest tertile: overall survival and time to locoregional failure

Table 1
Patient and tumor baseline characteristics (df = degrees of freedom; trend = across OPN groups)

	With OPN N=	OPN <407ng/mL lowest tertile	407<OPN<710ng/mL middle tertile	OPN >711ng/mL highest tertile	P value
All patients	578	191	194	193	
Age (median)	56	55	55	59	<0.001
Gender					
Male	501	158	170	173	0.046 (trend)
Female	77	33	24	20	
(% female)	13%	17%	12%	10%	
Primary site:					
Oropharynx	318	116	99	103	0.44 (6df)
Oral Cavity	81	26	29	26	
Larynx	105	32	36	37	
Hypopharynx	74	17	30	27	
T stage					
T1-2	100	39	42	19	0.006 (trend)
T3-4	478	152	152	174	
(% T3-4)	83%	80%	78%	90%	
N stage					
N0-1	159	45	52	62	0.060 (trend)
N2-3	419	146	142	131	
(% N2-3)	72%	76%	73%	68%	
ECOG					
0	361	124	129	108	0.069 (trend)
1, 2	217	67	65	85	
(% 1, 2)	38%	35%	34%	44%	
Haemoglobin					
Low	158	43	47	68	0.005 (trend)

	With OPN N=	OPN <407ng/mL lowest tertile	407<OPN<710ng/mL middle tertile	OPN >711ng/mL highest tertile	P value
High	420	148	147	125	0.21 (2df)
(% Low)	27%	23%	24%	35%	
Treatment arm					
No TPZ	295	102	89	104	0.21 (2df)
TPZ	283	89	105	89	
(%randomized to TPZ)	49%	47%	54%	46%	