Prognostic and Predictive Significance of Plasma HGF and IL-8 in a Phase III Trial of Chemoradiation with or without Tirapazamine in Locoregionally Advanced Head and Neck Cancer

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Abstract

Purpose: Hepatocyte growth factor (HGF) is a hypoxia-induced secreted protein that binds to cMet and regulates interleukin (IL)-8 expression. We evaluated the role of circulating HGF and IL-8 as prognostic and predictive factors for efficacy of tirapazamine (TPZ), a hypoxic cell cytotoxin.

Experimental Design: Patients with stages III to IV head and neck cancer were randomized to receive radiotherapy with cisplatin (CIS) or CIS plus TPZ (TPZ/CIS). Eligibility for the substudy included plasma sample availability for HGF and IL-8 assay by ELISA and no major radiation deviations (N = 498). Analyses included adjustment for major prognostic factors. p16INK4A staining (human papillomavirus surrogate) was carried out on available tumors. Thirty-nine patients had hypoxia imaging with 18F-fluoroazomycin arabinoside (18FAZA)–positron emission tomography.

Results: Elevated IL-8 level was associated with worse overall survival (OS) irrespective of treatment. There was an interaction between HGF and treatment arm (P = 0.053); elevated HGF was associated with worse OS in the control but not in the TPZ/CIS arm. Similar trends were observed in analyses restricted to p16INK4A-negative patients. Four subgroups defined by high and low HGF/IL-8 levels were examined for TPZ effect; the test for interaction with arm was P = 0.099. TPZ/CIS seemed to be beneficial for patients with high HGF and IL-8 but adverse for low HGF and high IL-8. Only HGF correlated with 18FAZA tumor standard uptake value.

Conclusions: IL-8 is an independent prognostic factor irrespective of treatment. There is an interaction between HGF and treatment arm. Certain subgroups based on IL-8/HGF levels seemed to do better with TPZ/CIS while others did worse, highlighting the complexity of hypoxia targeting in unselected patients.

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Introduction

Tumor hypoxia represents an imbalance between oxygen supply and consumption. Hypoxia has been shown to enhance radiation resistance and metastasis in solid tumors, including head and neck squamous cell carcinoma (HNSCC) (1, 2). Several approaches have been used to target tumor hypoxia with radiation therapy, including methods to increase tumor oxygen supply or reduce oxygen consumption, high linear energy transfer radiation, hypoxic cell radiosensitizers, or hypoxic cell cytotoxins (3, 4). Although several strategies were found promising in early phase II studies, many failed when tested in large randomized trials. Failure in hypoxia targeting could partially be attributed to poor patient selection for such therapies (5). Therefore, it is important to identify markers that can be used to select for patients who would most benefit from a hypoxia-targeting approach.

Several molecular markers are induced by hypoxia, among which are the hepatocyte growth factor (HGF), its receptor cMet and one of its downstream effectors, interleukin (IL)-8. Physiologically, the HGF/cMet pathway plays a major role in organ development and normal tissue homeostasis (6–8). However, inappropriate activation of this pathway is linked to tumor transformation, growth,
Preclinical studies indicate that the hypoxic tumor microenvironment can be exploited for therapeutic benefit. The presence of tumor hypoxia is consistently associated with an adverse prognosis in head and neck cancers (HNC). Despite the importance of this microenvironment factor, the results of trials targeting hypoxia in unselected patient populations have been disappointing. Here, we measured the pretreatment plasma levels of both cytokines in patients from TPZ and conversely a population that does not benefit from either cMet short hairpin RNA or inhibitors resulted in decreased IL-8 level (11, 18, 19). Although the complete mechanism of hypoxia induction of IL-8 has not been clearly delineated, the HGF/cMet pathway may mediate part of this effect. Therefore, both HGF and IL-8 are attractive secreted molecules to evaluate as potential predictive markers for hypoxia-directed therapy.

Tirapazamine (TPZ) was developed as a hypoxic cell cytotoxin that can be administered concurrently with chemoradiation therapy (CRT). The TROG-02.02 (HeadSTART) trial was a large international phase III trial, which showed that the addition of TPZ did not improve survival than those with HPV(−) tumors (29). To address the effect of HPV, we also stratified a subset of patients by p16INK4A status (an HPV surrogate marker) and evaluated the impact of HGF and IL-8 in p16INK4A(+) and p16INK4A(−) patients. 18F-fluoroazomycin arabinoside (18FAZA) is a novel hypoxia tracer used in positron emission tomography (PET) imaging and has been shown to predict the success of TPZ in combination with CRT in animal models (30–32). It has been used to image hypoxia in human HNSCC (31). In a small subset of patients enrolled in the TROG-02.02 trial, we carried out pretreatment 18FAZA imaging and correlated tumor 18FAZA standard uptake values (SUV) with pretreatment HGF and IL-8 levels.

Materials and Methods

Study design

We have previously reported on the results of this trial, eligibility criteria, treatment details, follow-up, and outcomes of the TROG-02.02 trial (20). For this substudy, additional eligibility criteria were available plasma samples for cytokine assay and no major radiation deviations predicted to have an impact on tumor control. Using plasma samples from the same patient population, we have also measured the levels of circulating osteopontin and VEGF, 2 other hypoxia-induced secreted markers, and have reported the results for osteopontin in a separate manuscript (33). Here, we focus on HGF and IL-8 as independent hypoxia markers. All studies were approved by the Local Institutional Review Board.

Endpoints

The primary endpoint was overall survival (OS) adjusted for the following prognostic factors: site (oropharynx/larynx vs. oral cavity/hypopharynx), T category (T1–2 vs. 3–4), N category (N0–1 vs. 2–3), hemoglobin (high vs. low), and Eastern Cooperative Oncology Group (ECOG) performance status (0 vs. 1–2). This same endpoint was defined for the main analysis (20). Another evaluated endpoint was failure-free survival (FFS).

HGF measurement

Standardized plasma collection, isolation, and storage procedures were used by all sites. Peripheral blood samples were collected in 5 to 10 mL EDTA tubes pretreatment. Plasma was isolated, aliquoted, and stored at −80°C until assayed by a central site (Amgen). A fibrinogen assay was used to ensure that all tested samples were plasma and not serum. HGF measurement was carried out with a modified ELISA system (R&D Systems) in which Assay Diluent GF2 (Meso Scale Discovery) was used to ensure assay linearity (34). We initially prospectively validated the prognostic value of HGF with a test set and validating set approach. We measured HGF level in an initial 168 patients, who were balanced for clinical characteristics. Upon determining that...
a higher HGF level was associated with worse survival in the test group, we validated its prognostic significance in the remaining patients (data not shown).

**IL-8 measurements**

IL-8 was quantified by ELISA (R&D Systems) per the manufacturer’s instruction at the Peter MacCallum Cancer Center.

**p16INK4A immunohistochemistry**

p16INK4A immunohistochemistry was conducted and quantified as previously described (29). p16INK4A staining intensity was scored as 0 (none), 1 (weak), 2 (moderate), or 3 (strong), with 0–1 defined as negative and 2–3 defined as positive (29).

**18Fluorodeoxyglucose and 18FASA–PET**

18Fluorodeoxyglucose (18FDG) and 18FAZA synthesis and imaging were conducted as described (31). Briefly, at 1 hour after FDG injection or 2 hours after FAZA injection (5.2 MBq/kg), a static PET acquisition was carried out and reconstructed (31). 18FAZA images were coregistered with 18FDG PET images using a mutual information algorithm and the 18FDG PET was then used to identify tumor and involved nodal areas as regions of interest for 18FAZA analysis. The maximum SUVs for 18FAZA and 18FDG were calculated separately for the tumor and the involved nodes using the RT_Image software (35).

**Statistical methods**

The distributions of marker variables according to baseline factors, and correlations between markers and SUVs were assessed with exact nonparametric tests (Wilcoxon, Fisher, and Spearman). The Kaplan–Meier method was used to estimate OS curves. OS was measured from the end of radiotherapy because of the radiation deviation eligibility criterion. The groups were compared with respect to OS, using the log-rank test and Cox proportional hazards model, adjusting for previously identified prognostic factors described above. We decided a priori to analyze HGF and IL-8 as dichotomous variables (median cut points). However, we also evaluated the markers as continuous variables. Median levels of HGF and IL-8 were calculated on all patients assayed with the particular marker. HRs for 2-group comparisons refer to high (>median):low (<median) for marker comparisons and TPZ:CIS for treatment arm comparisons. To identify patient subgroups based on the 2 markers, such identification was guided by tests for

<table>
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<th>Without HGF/IL-8 N = 195</th>
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<th>HGF high (&gt;median) N = 221</th>
<th>IL-8 low (&lt;median) N = 255</th>
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<sup>a</sup>P < 0.05.

<sup>b</sup>Hemoglobin ≤13.5 g/dL for men and ≤12.5 g/dL for women.
statistical interaction between treatment of the 2 arms and the 2 markers with 3 degrees of freedom. All P values are 2-sided. Statistical analyses were conducted with the R statistical package (36).

Results

Patient characteristics

Of 853 eligible patients, 596 patients had plasma available for HGF and IL-8 assays. Ninety-eight patients were excluded because of major radiotherapy deviations, leaving 498 for marker analysis (Supplementary Table SA). Of these, 165 patients had died, 199 had failed or died, and 111 had experienced locoregional failure as a first failure. Table 1 shows the characteristics of the radiation compliant patients who did and did not have marker data; those with marker data available had lower hemoglobin, more laryngeal, and fewer hypopharyngeal cancers.

The median HGF level was 823 pg/mL (range, 190–42,300) and the median IL-8 level was 6.48 pg/mL (range, 0–365). Table 1 also shows the distribution for the low (<median) versus high (≥median) HGF and IL-8 groups, respectively. High HGF level was significantly associated with higher T-stage (T3–4), worse performance status (ECOG PS > 0), more oral cavity and hypopharyngeal primaries, and being a current smoker; whereas high IL-8 was only associated with higher T-stage. Consistent with the known biological association, there was a correlation between HGF and IL-8; the patient distribution for the marker groups was 35% for both markers being low, 28% for both being high, 16% for IL-8–low/HGF-high and 21% for IL-8–high/HGF-low group (P < 0.001).

In the cohort with known p16INK4A status (n = 223), there was a correlation between p16INK4A and HGF levels (P = 0.001) and between p16INK4A and IL-8 levels (P = 0.003), with high levels of each marker being more common in p16INK4A(−) patients (Supplementary Table SB).

Treatment outcomes

Both pretreatment plasma HGF and IL-8 levels were prognostic for OS and FFS on univariate analysis in the whole population. For HGF, the HR was 1.50 (P = 0.008) for OS and 1.43 (P = 0.011) for FFS when analyzed as a dichotomous variable (by the median), and 1.42 (per doubling; P = 0.001) for OS and 1.39 (P = 0.001) for FFS, respectively, when evaluated as a continuous variable (log transformed). For IL-8, the HR was 1.86 (P < 0.001) for OS and 1.59 (P = 0.001) for FFS when analyzed as a dichotomous variable (by the median) whereas it was 1.12 (per doubling; P = 0.002) for OS and 1.08 (P = 0.013) for FFS, respectively, when assessed as a continuous variable (log transformed).

However, when these analyses were repeated adjusting for known prognostic factors, to address the main aims of the study, only IL-8 remained significant: the HR for HGF was 1.20 (P = 0.27) and for IL-8 was 1.55 (P = 0.007; Supplementary Figs. SA and SB). However, there was an interaction between HGF and treatment arm (Pinteraction = 0.053). High HGF levels predicted for worse OS in the control arm but not in the TPZ/CIS arm (Fig. 1). The 2 year
OS on the control arm was 63% for the high HGF versus 76% for the low HGF group (HR = 1.62, p = 0.028) (Table 2). On the TPZ/CIS arm, the 2 year OS was 72% versus 69% for high and low HGF, respectively (HR = 0.84, p = 0.46). In contrast, there was no interaction between IL-8 (analyzed by median) and treatment (p = 0.66). High IL-8 level was associated with worse OS, regardless of the treatment received (Fig. 2).

As there was an interaction between HGF and arm, we examined the effect of treatment in the high and low HGF groups, adjusting for prognostic factors using a 3-degree of freedom test. There was a suggestion that TPZ/CIS may be associated with an adverse outcome in low HGF patients and a better outcome in high HGF patients, but these differences were not statistically significant. Within the low HGF group the 2-year OS was 76% for CIS versus 69% for the TPZ/CIS (HR = 1.43, P = 0.12), and within the high HGF group the 2-year OS was 63% for CIS versus 72% for TPZ/CIS (HR = 0.76, P = 0.21; Table 2).

We next sought to determine whether there was an interaction between the 4 possible high/low IL-8/HGF combinations and treatment arm. The P value for this interaction adjusting for prognostic factors was 0.099. Although not statistically significant, the P value was small enough to suggest that there may be differences in the relative efficacy of TPZ among the 4 subgroups. Based on this, we proceeded to see whether there were any significant differences by arm within the IL-8/HGF subgroups (Fig. 3A, interaction analysis; Table 2). Although none of the tests was significant, the HRs for 2 of the groups were large and in opposite directions. This suggests that TPZ/CIS may be advantageous in the IL-8-high/HGF-high group (HR = 0.64, P = 0.12) and adverse in the IL-8-high/HGF-low group (HR = 1.86, P = 0.07; Fig. 3B).

We also evaluated the prognostic importance of HGF by p16INK4A status. Of 498 patients, 223 patients had tissue slides available and analyzable for p16INK4A; 95 tumors were p16INK4A(-) and 128 were p16INK4A(+). With the caveat that these are small cohorts, there was no apparent difference in outcomes by HGF level for either arm in the p16INK4A(-) patients (Supplementary Fig. SC), while in the p16INK4A(+) patients, there was a trend for worse OS with high HGF level in the control but not in the TPZ/CIS arm (Supplementary Table 2).

<table>
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<th>Deaths/N</th>
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<td>HGF</td>
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NOTE: All analyses except for those conducted on p16 subgroups are adjusted for the prognostic factors. HGF(low), HGF-low; HGF(high), HGF-high; IL-8(low), IL-8-low; IL-8(high), IL-8-high.

Abbreviation: OP, oropharynx.

*HR comparing HGF-high with HGF-low, IL-8(low) with IL-8(high), or TPZ/CIS with CIS.
The 2-year OS on the control arm was 52% (high HGF) versus 68% (low HGF); HR = 1.90, \( P = 0.099 \). The \( P \) value of the test of interaction between HGF and treatment in the \( p16^{INK4A}(-) \) patients was 0.15.

Correlation between PET imaging and HGF/IL-8 levels

Thirty-nine patients from the Peter MacCallum Cancer Centre also had pretreatment \( ^{18}F \)FZA hypoxia PET imaging together with plasma HGF and IL-8. HGF levels significantly correlated with the maximum \( ^{18}F \)FZA SUV and \( ^{18}F \)FDG SUV (SUV\(_{max}\)) in the primary tumor (Table 3). No correlation was noted for IL-8 with any \( ^{18}F \)FZA or \( ^{18}F \)FDG parameters.

Discussion

Despite extensive knowledge of tumor hypoxia, attempts to target it, including TPZ, have not been successful in large phase III trials (3, 20, 37–41). This may be due to inappropriate patient selection, hence diluting the patient pool with individuals who would not benefit and may even be harmed by the drug (42). One HNSCC patient group that may not benefit is the one with HPV(+) oropharyngeal carcinoma. These patients are known to have an excellent prognosis with conventional therapy and considerations are being made to deintensify their treatment. A prior study has shown that they did not benefit from nimorazole, a hypoxic cell radiosensitizer (43). These patients made up a large percentage of the patients in the TROG-02.02 trial and may partially explain the negative results here. Subset analysis showed a trend for improved locoregional control with TPZ in the HPV(-) patients (29). Therefore, it is crucial to identify molecular predictors for hypoxia-targeted therapy.

Because both HGF and IL-8 can be induced by hypoxia, we investigated the prognostic and predictive significance of these markers on TPZ efficacy in this randomized study. We found that IL-8 was a prognostic factor for survival in both arms, whereas there was an interaction between HGF and treatment with elevated HGF level being associated with worse OS in the control but not in the TPZ/CIS arm. Within the high HGF group, there was a trend for better OS favoring TPZ/CIS; however the \( P \) value did not reach statistical significance. When HGF and IL-8 were combined, the patients who had the largest hazard reduction of death with the addition of TPZ were those with elevated pretreatment level of both markers. In contrast, patients with high IL-8 but low HGF level had worse survival on TPZ/CIS. The reason for the differential impact of TPZ on these 2 patient groups is unclear though may be related to IL-8 regulation. Hypoxia can induce both markers with HGF induction occurring before IL-8 (44). HGF can directly promote IL-8 expression in HNSCC through both mitogen-activated protein/extracellular signal-regulated kinase (MEK)- and phosphoinositide 3-kinase–dependent pathways (18). Although HGF is secreted primarily from HNSCC tumor–derived fibroblasts, IL-8 is often produced by tumor cells (11, 18). In addition to being regulated by hypoxia and HGF, tumor IL-8 expression can be affected by other factors, including inflammation, oxidative stress, the Src kinases, and the NF-\( \kappa \)B/AP1 pathway (45, 46). We hypothesize that patients with high IL-8 level belong to 2 different groups:
those with IL-8 being induced by hypoxia via stromal-derived HGF (IL-8–high/HGF-high) and those with IL-8 being promoted by factors unrelated to hypoxia (IL-8–high/HGF-low). Because hypoxia is a major driver of tumor progression in the first group, targeting hypoxia with TPZ would be beneficial here. In contrast, because hypoxia is unlikely to be the cause of tumor aggression in the second group, the use of TPZ would not benefit these patients. Moreover, these patients, while deriving no benefit from TPZ, may be adversely affected by the lower dose of CIS in the TPZ/CIS arm (75 mg/m² in TPZ/CIS vs. 100 mg/m² in the control arm). These data suggest that biomarker-guided
misonidazole (18FMISO), a closely related compound to FDG PET in 39 patients with one or both markers

<table>
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<th>Marker</th>
<th>Location</th>
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<th>FDG Spearman correlation</th>
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</tbody>
</table>

In summary, we found that IL-8 is an independent prognostic factor irrespective of treatment, and that there is an interaction between treatment arm and HGF level. Such an interaction means that the arms must differ for patients with low HGF or for patients with high HGF or for both. The examination of the differences between arms within the HGF subgroups provided no definitive answer as to where these differences may lie but indicated that either TPZ/CIS is superior for high HGF or TPZ/CIS is inferior for low HGF or both are true. Furthermore, using a combination of IL-8 and HGF, our data suggested that the patients with IL-8–high/HGF-high may benefit from TPZ/CIS whereas those with IL-8–high/HGF-low may do better with standard treatment. These biomarker-defined groups highlight the complexity of conducting hypoxia-targeted trials in unselected patients and suggest that prospective collection of blood and tumor samples for biomarker validation is critical for success of future hypoxia-targeted strategies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Prognostic and Predictive Significance of Plasma HGF and IL-8 in a Phase III Trial of Chemoradiation with or without Tirapazamine in Locoregionally Advanced Head and Neck Cancer

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