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Expression of the c-Met oncogene by tumor cells predicts a favorable outcome in classical Hodgkin’s lymphoma

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ABSTRACT

Background
The c-Met signaling pathway regulates a variety of biological processes, including proliferation, survival and migration. Deregulated c-Met activation has been implicated in the pathogenesis and prognosis of many human malignancies. We studied the function and prognostic significance of c-Met and hepatocyte growth factor protein expression in patients with classical Hodgkin’s lymphoma.

Design and Methods
Expression of c-Met and its ligand, hepatocyte growth factor, were determined by immunohistochemistry. Prognostic values were defined in cohorts of German and Dutch patients with classical Hodgkin’s lymphoma. Functional studies were performed on Hodgkin’s lymphoma cell lines.

Results
Expression of c-Met was detected in the tumor cells of 52% (80/153) of the patients and expression of its ligand, hepatocyte growth factor, in 8% (10/121) of the patients. c-Met expression correlated with a 5-year freedom from tumor progression of 94%, whereas lack of expression correlated with a 5-year freedom from tumor progression of 73% (P<0.001) in the combined cohort. In multivariate analysis both c-Met (hazard ratio 5.0, 95% confidence interval 1.9-13.3, P<0.001) and stage (hazard ratio 2.8, 95% confidence interval 1.2-6.4, P=0.014) were independent predictors for freedom from tumor progression. In functional studies activation with hepatocyte growth factor did not affect cell growth, while the c-Met inhibitor SU11274 suppressed cell growth by inducing G2/M cell cycle arrest.

Conclusions
Although functional studies showed an oncogenic role of the hepatocyte growth factor/c-Met signaling pathway in cell cycle progression, expression of c-Met in tumor cells from patients with classical Hodgkin’s lymphoma strongly correlated with a favorable prognosis in two independent cohorts.

Key words: c-Met, classical Hodgkin’s lymphoma, hepatocyte growth factor, prognosis.


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Introduction

Hodgkin’s lymphoma is a B-cell neoplasm characterized by a minority of neoplastic cells within an extensive infiltrate of reactive cells. There is a classical form and a nodular lymphocyte predominant variant. The tumor cells in classical Hodgkin’s lymphoma (cHL), the so-called Hodgkin and Reed-Sternberg (HRS) cells, are derived from pre-apoptotic germinal center B cells that acquire crippling immunoglobulin gene mutations and/or lose their capacity to express a high affinity B-cell receptor.\(^1\) Epstein-Barr virus (EBV) infection, constitutive activation of nuclear factor \(\kappa\)B and aberrant activation of receptor tyrosine kinases contribute to the survival and proliferation of HRS cells.\(^2,3\)

The prognosis of cHL patients improved greatly over the last three decades and current treatment regimens have reduced treatment failure to less than 20\% even in advanced stages.\(^4\) However, long-term toxicities and especially secondary malignancies are major concerns and the main challenge in cHL is to avoid overtreatment in individual patients. The Ann Arbor staging system is used to classify cHL into early or advanced stage disease and treatment is tailored accordingly. In addition, several other clinicopathological factors are used for risk stratification.\(^5\)

Although some prognostic tissue markers, such as number of tumor-infiltrating macrophages,\(^6\) HLA class II expression by tumor cells\(^,\) and tumor cell EBV status,\(^,\) have been published, there is currently no pre-treatment cHL prognostic factor that is applied for prediction of treatment response in a clinical setting.

The hepatocyte growth factor (HGF)/c-Met signaling pathway regulates a variety of biological processes, including proliferation, survival and migration.\(^7\) Deregulated c-Met activation, caused by gene amplification, translocation, mutation or autocrine/paracrine HGF signaling, has been implicated in the pathogenesis of many human cancers.\(^8\) Furthermore, c-Met is of prognostic significance in numerous malignancies, such as diffuse large B-cell lymphoma,\(^9\) bladder cancer,\(^10\) breast cancer,\(^11\) colorectal cancer\(^12\) and ovarian cancer.\(^13\) Inhibitors have been developed to target this signaling pathway and one of these, SU11274,\(^14\) shows effective inhibition of the c-Met signaling pathway, thereby affecting the survival and growth of lung cancer,\(^15\) mesothelioma,\(^16\) and melanoma\(^17\) cell lines.

c-Met expression has been reported in HRS cells in tissue samples from 53\% (6/18)\(^20\) and 100\% (57/57)\(^21\) of cHL patients. HGF expression was observed in infiltrating cells, especially in dendritic cells, but not in tumor cells.\(^21\) Additionally, both c-Met and HGF are expressed in some cHL cell lines.\(^22,23\) We studied the function of the HGF/c-Met pathway in cHL cell lines and determined the prognostic value of c-Met expression in two independent cohorts of patients.

Design and Methods

Patients and tissue data

Primary cHL tissues were retrieved from the Department of Pathology, University Medical Center Groningen, the Netherlands (\(n=62\), collected from 1993 up to 2009) and from the Institute of Pathology, University of Ulm, Germany (\(n=91\), collected from 1994 up to 2009).

Almost all patients were treated and staged according to pediatric or adult clinical trial protocols (EORTC or GHSG). The basic characteristics of the German and the Dutch patients are presented in Table 1. Information on the presence of B symptoms was not available for two patients and data were lacking on the presence of bulky disease for four patients; these patients were excluded from the respective analyses. The International Prognostic Score (IPS), which incorporates information on age, stage, sex, hemoglobin and albumin levels and leukocyte and lymphocyte counts, was applied only in patients with advanced stage (stages III and IV). The IPS was unknown for four patients and these patients were excluded from the comparisons between c-Met expression and IPS. No significant differences regarding age at diagnosis, sex, histological subtype, stage of disease and EBV status were observed between the cohorts. The median follow-up was 61 months for the German cohort (interquartile range, 39-87 months) and 44 months for the Dutch cohort (interquartile range, 22-83 months). The German cohort differed significantly from the Dutch cohort by treatment modality (more German patients received the BEACOPP regimen and combined modality treatment was more often used) (Table 1). The study protocol was consistent with international ethical and professional guidelines (the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice).

Immunohistochemistry

Immunohistochemistry (IHC) was performed with antibodies against c-Met (C-28) (Santa Cruz Biotechnology, Santa Cruz, CA, http://www.scbt.com) and HGF (B-8, Santa Cruz) and scored positive or negative.

Table 1. Basic characteristics of the German, Dutch and total combined cohorts of patients. The \(P\) value refers to the difference between the German and the Dutch patients.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>All ((n=153))</th>
<th>German ((n=91))</th>
<th>Dutch ((n=62))</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (range)</td>
<td>29 (7-75)</td>
<td>29 (8-75)</td>
<td>28 (7-66)</td>
<td>0.75</td>
</tr>
<tr>
<td>Male</td>
<td>81 (53)</td>
<td>50 (55)</td>
<td>31 (50)</td>
<td>0.62</td>
</tr>
<tr>
<td>Histology subtype</td>
<td></td>
<td></td>
<td></td>
<td>0.39</td>
</tr>
<tr>
<td>Nodular sclerosis</td>
<td>110 (72)</td>
<td>65 (71)</td>
<td>45 (73)</td>
<td></td>
</tr>
<tr>
<td>Mixed cellularity</td>
<td>23 (15)</td>
<td>15 (17)</td>
<td>8 (13)</td>
<td></td>
</tr>
<tr>
<td>Lymphocyte rich</td>
<td>3 (2)</td>
<td>3 (3)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>Lymphocyte depleted</td>
<td>1 (1)</td>
<td>-</td>
<td>1 (2)</td>
<td></td>
</tr>
<tr>
<td>cHL, NOS</td>
<td>16 (11)</td>
<td>8 (9)</td>
<td>8 (13)</td>
<td></td>
</tr>
<tr>
<td>EBV status</td>
<td></td>
<td></td>
<td></td>
<td>0.17</td>
</tr>
<tr>
<td>Positive</td>
<td>35 (23)</td>
<td>17 (19)</td>
<td>18 (28)</td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>118 (77)</td>
<td>74 (81)</td>
<td>44 (71)</td>
<td></td>
</tr>
<tr>
<td>Ann Arbor stage</td>
<td></td>
<td></td>
<td></td>
<td>0.62</td>
</tr>
<tr>
<td>I-II</td>
<td>87 (57)</td>
<td>50 (55)</td>
<td>37 (60)</td>
<td></td>
</tr>
<tr>
<td>III-IV</td>
<td>65 (42)</td>
<td>40 (44)</td>
<td>25 (40)</td>
<td></td>
</tr>
<tr>
<td>n.a.</td>
<td>1 (1)</td>
<td>1 (1)</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

**Treatment:** \(< 0.001\)

> Combined modality:

| ABVD + RT | 47 (31) | 31 (34) | 16 (26) | |
| BEACOPP + RT | 16 (11) | 16 (18) | - | |
| BEACOPP, ABVD + RT | 3 (2) | 3 (3) | - | |
| Pediatric protocols + RT | 19 (12) | 7 (8) | 12 (19) | |
| COPP/ABVD + RT | 3 (2) | 3 (3) | - | |
| MOPP/ABV + RT | 2 (1) | - | 2 (3) | |

> Chemotherapy alone:

| ABVD | 27 (18) | 6 (7) | 21 (34) | |
| BEACOPP | 27 (18) | 24 (26) | 3 (5) | |
| MOPP/ABV | 7 (5) | - | 7 (11) | |
| Other | 1 (1) | 1 (1) | - | |

> Radiotherapy alone:

| 1 (1) | - | 1 (2) | |

CHL, classical Hodgkin’s lymphoma; n.o.t., not otherwise specified; n.a.: not available; RT, radiotherapy (involved field or involved node)
USA), HGF (R&D Systems, Minneapolis, MN, USA) and CD30 (Dako, Glostrup, Denmark) on paraffin-embedded tissue sections after antigen retrieval (pH 9). Staining was visualized using horseradish peroxidase-labeled secondary antibodies and 3,3’-diaminobenzidine (Sigma Aldrich, St Louis, MO, USA). Appropriate positive and negative controls were performed for each staining. The Dutch cases were stained and scored on a tissue micro-array (TMA) or on whole tissue sections and the German cases were scored on whole tissue sections. For c-Met all 153 cases were stained and for HGF all German cases and the Dutch TMA were stained (121 cases). Each case in the TMA was represented by two tissue cores and cases were scored only if at least ten tumor cells were present in both cores. CD30 expression was used to identify the tumor cells. The median number of tumor cells for each case was 30. Only staining of the tumor cells was scored. The distribution of percentages of c-Met-positive tumor cells showed a distinction at 30% (20% or less: 72/153 and 30% or more: 80/153). We hypothesized that this distinction reflects two biologically different subsets and a cut-off of ≥ 30% positive HRS cells was chosen. The percentage of HGF-positive cells for most patients was 10% or less (49/153) and a cut-off of ≥ 20% of positive HRS cells was chosen.

Cell lines

The CHL cell lines L428, L1236, KMH24 and U-HO125 were cultured in RPMI-1640 medium supplemented with ultraglutamine-1, 100 U/ml penicillin/streptomycin and 10% fetal calf serum (5% from 500 ng of total RNA in 20 μl of the L428 cell line) (Lonza Walkersville, Walkersville, MD, USA).

Enzyme-linked immunosorbent assay on the supernatant of cultures of classical Hodgkin’s lymphoma cell lines

Levels of HGF protein were measured in cell culture supernatants by an enzyme-linked immunosorbent assay according to the protocol provided by the manufacturer (R&D Systems).

Quantitative reverse transcriptase polymerase chain reaction

RNA was extracted using Trizol® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s protocol. cDNA was made from 500 ng of total RNA in 20 μl of reactions using Superscript II and random primers (Invitrogen). Two nanograms of cDNA were used in the quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) in triplicate using Sybergreen (for HGF) and probe (for U6), as described by the manufacturer (Applied Biosystems, Foster City, CA, USA). The primer sequences used for the amplification were as follows: U6 forward primer: 5’-ttggtgagcttataacta-3’ and reverse primer 5’-aatggaaacctcagaa-3’; U6 probe: 5’-ccctgtgcaagttgag-3’, HGF forward primer (exon 5): 5’-caactccagaggttga-3’ and reverse primer (exon 6) 5’-acctccctggctgcat-3’. U6 was used for normalization (ΔCt = CtU6 - CtHGF). Relative expression levels of HGF were determined using the formula 2^ΔCt.

Western blot

Cell lysates were separated on polyacrylamide gels and electroblotted onto nitrocellulose membranes using standard protocols. Blots were incubated with primary antibodies, c-Met (C12, Santa Cruz), p-Met (Tyr1234/1235), p-p44/42 MAPK (Thr202/Tyr204) (20G11), and p-Akt (Ser473) (D9E) (Cell Signaling Technology, Boston MA, USA), at 4°C overnight. Immunostaining was amplified by incubation with horseradish peroxidase-conjugated antibodies and chemiluminescence was detected with ECL (Pierce, Rockford, USA).

MTT assay

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma Aldrich) was added to cells and incubated for 4 h at 37°C. The cells were resolved in dimethylsulfoxide (Sigma Aldrich) and absorption was measured at 540 nm.

Cell cycle analysis

Hypotonic DNA staining buffer (0.1% sodium citrate; 0.3% Triton-X 100; 0.01% propidium iodide, 0.002% ribonuclease A) was added to the cells and mixed well. Data were acquired with a flow cytometer (Calibur, BD Biosciences, San Jose, CA, USA).

Definition of endpoints

Freedom from tumor progression (FFTP), and overall survival were the end-points for this study. FFTP was calculated from the date of diagnosis until the date of a disease-related event. Disease-related events were defined as progression during treatment, death during treatment with unknown disease status, less than a complete remission after treatment and relapse after treatment, including death due to lymphoma progression. In case none of these events occurred, patients were censored at the date of last follow-up or death. Overall survival was defined as the date of diagnosis until date of death. In the case of no events, patients were censored at the date of last follow-up.

Statistical analysis

Fisher’s exact test was used for the analysis of differences in patients’ characteristics between the German and Dutch cohorts of patients and between the c-Met-positive and c-Met-negative patients. Survival curves were computed using the method of Kaplan and Meier. Differences between the curves were calculated using the log-rank statistic. Univariate and multivariate Cox logistic regression models were applied to identify prognostic factors for survival. SPSS statistical software version 18.0 (SPSS Inc., Chicago, IL, USA) was used.

Results

c-Met and hepatocyte growth factor expression in chronic Hodgkin’s lymphoma tissue

Cytoplasmic and membranous c-Met expression in more than 30% of the tumor cells was observed in 49 out of 91 German patients (54%) and 31 out of 62 Dutch patients (50%) (Figure 1A and Online Supplementary Table S1). Expression was not significantly different between the two cohorts. Although c-Met expression could be detected in the reactive cellular background, no specific pattern of c-Met expression was observed between tumor cells and infiltrating cells.

HGF staining in more than 20% of the tumor cells was detected in 6 out of 91 German patients (7%) and 4 out of 30 Dutch patients (13%) (Figure 1B and Online Supplementary Table S1). Expression was not significantly different between the two cohorts. Co-expression of c-Met and HGF in tumor cells was found in only 4 of 121 CHL cases (3%) (Online Supplementary Table S2). Reactive cells stained positive in all CHL cases, but no HGF staining was observed in the lymphocytes directly surrounding the tumor cells.

Expression of c-Met (52% positive in the total cohort) did not correlate with age, sex, histological subtype, stage of disease, EBV status (Online Supplementary Table S2) or other known clinical prognostic factors (erythrocyte sedi-
Prognostic significance of c-Met expression

In both the German and the Dutch cohorts, c-Met-positive patients had a significantly more favorable 5-year FFTP compared to c-Met-negative patients (Figure 2A,B). Univariate Cox regression analysis in the German cohort showed that lack of c-Met expression correlated with a hazard ratio (HR) of 4.2 ($P=0.013$, 95% CI 1.4-13.2), and stage with a HR of 4.6 ($P=0.008$, 95% CI 1.5-14.4). In multivariate analysis both stage (HR 4.5, $P=0.010$, 95% CI 1.5-13.9) and c-Met (HR 3.9, $P=0.018$, 95% CI 1.3-12.3) remained independent predictors for FFTP. In the Dutch cohort, c-Met expression predicted a favorable outcome in cHL.

Figure 1. c-Met and HGF expression in cHL tissue. (A) Representative cHL case showing expression of c-Met in HRS cells (arrow). Expression of c-Met in the reactive cells can also be observed. (B) Representative cHL case showing expression of HGF in HRS cells (arrows). Reactive cells were also positive for HGF.

Figure 2. Kaplan-Meier estimates for freedom from tumor progression (FFTP) and overall survival (OS) according to c-Met expression. c-Met positivity of HRS cells correlated with very favorable FFTP in both the German (A) and the Dutch (B) cohorts. In the combined cohort, c-Met expression again correlated with favorable FFTP (C) and OS (D) and was independent of disease stage (E and F). Especially among patients with advanced stage disease (F), absence of c-Met correlated with a low 5-year FFTP rate (57%) compared to the rate in patients who were c-Met-positive (91%).
In the combined cohort, c-Met expression correlated with a 5-year FFTP of 94%, whereas no expression correlated with a 5-year FFTP of 73% (log rank P<0.001, Figure 2C). Univariate Cox regression analysis showed that lack of c-Met expression correlated with a HR of 5.1 (P<0.001, 95% CI 1.9-13.5) compared to positive expression (Table 2). Stage was the only other factor (HR 2.8, P=0.012) that could significantly predict FFTP. In multivariate analysis both c-Met (HR 5.0, P<0.001) and stage (HR 2.8, P=0.014) remained independent predictors for FFTP. Especially in patients with advanced disease, lack of c-Met expression correlated with a very poor 5-year FFTP rate (57% versus 91% in c-Met-positive patients; Figure 2E, F).

In the combined cohort, 5-year overall survival rates were 98% and 84% for patients expressing or not expressing c-Met, respectively (log rank P=0.006, Figure 2D). For HGF, no significant differences were observed in FFT and overall survival.

**The c-Met/hepatocyte growth factor signaling pathway in classical Hodgkin’s lymphoma cell lines**

High c-Met expression was only observed in the L428 cell line (Figure 3A). HGF protein levels were high in the cell culture supernatant of KMH2, with corresponding levels of HGF mRNA (Figure 3B, C).

Phosphorylated c-Met (p-Met) was present only in the L428 cell line and stimulation by HGF strongly increased p-Met levels in this cell line. Low levels of p-Met were induced in L1236 and U-HO1 (Figure 3D). Analysis of p-Met downstream substrates in the L428 cell line showed up-regulation of p-Akt and p-Erk1/2 upon HGF stimulation and this up-regulation was effectively blocked by SU11274 in a dose-dependent manner (Figure 3E). HGF stimulation did not affect cell growth (data not shown), whereas SU11274 significantly suppressed cell growth in a dose-dependent manner (Figure 3F). This suppression of cell growth was not caused by an induction of apoptosis (data not shown). Cell cycle analysis showed that the proportion of cells in the G2/M phase increased from 15% in control cells to 52% in cells treated with SU11274 (2.5 μM) (P<0.05) (Figure 3G). This effect was greater at a higher concentration (5 μM) (data not shown) or after longer incubation (48 h or 72 h) (Figure 3G), indicating dose- and time-dependent effects. After 48 h and 72 h a population of tetraploid cells appeared (Figure 3G).

**Table 2. Univariate and multivariate Cox regression analyses for freedom from tumor progression for c-Met expression and other clinical prognostic factors in the combined cohort.**

<table>
<thead>
<tr>
<th>Combined cohorts</th>
<th>N</th>
<th>Univariate</th>
<th></th>
<th>Multivariate</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>c-Met expression &lt; 30%</td>
<td>73</td>
<td>5.1</td>
<td>1.9-13.5</td>
<td>0.001</td>
<td>5.0</td>
</tr>
<tr>
<td>Stage III/IV</td>
<td>65</td>
<td>2.8</td>
<td>1.2-6.3</td>
<td>0.014</td>
<td>2.8</td>
</tr>
<tr>
<td>IPS = 3</td>
<td>35</td>
<td>2.1</td>
<td>0.7-6.9</td>
<td>0.201</td>
<td></td>
</tr>
<tr>
<td>B-symptoms present</td>
<td>70</td>
<td>1.8</td>
<td>0.8-3.0</td>
<td>0.166</td>
<td></td>
</tr>
<tr>
<td>Bulky disease</td>
<td>46</td>
<td>0.9</td>
<td>0.7-1.3</td>
<td>0.638</td>
<td></td>
</tr>
</tbody>
</table>

HR: hazard ratio; CI: confidence interval; IPS: International Prognostic Score.
c-Met signaling pathway in the cHL cell line L428. Although p-Met, p-Akt and p-Erk1/2 are strongly up-regulated upon stimulation with HGF, no effect was observed on cell growth in L428 cells. Nevertheless, we did observe an inhibitory effect on cell growth with c-Met inhibitor SU11274, which blocked the constitutive phosphorylation of c-Met and downstream kinases and induced G2/M cell cycle arrest.

The stimulating effects of c-Met on cell cycle progression in L428 cells are inconsistent with the favorable prognostic value of c-Met positivity in HRS cells in cHL patients. The unique histological phenotype of cHL might be a key factor in this unexpected positive prognostic effect. The proportion of tumor cells in cHL is small, typically less than 1%, and these cells are located within an extensive infiltrate of reactive cells. In comparison, the proportion of tumor cells in diffuse large B-cell lymphoma and solid malignancies is generally between 40% and 80%. When focusing on the infiltrating cells, several known effects of c-Met may explain the favorable effect of c-Met in Hodgkin’s lymphoma. In a rat model of cirrhosis, c-Met strongly reduced liver fibrosis and decreased transforming growth factor-β (TGF-β) levels. Since TGF-β plays an important role in suppression of an effective immune response in cHL, the activation of c-Met in HRS cells could result in decreased TGF-β production and, thereby, in enhanced anti-tumor responses. In monocytes, activation of c-Met induced up-regulation of pro-inflammatory cytokines and a pro-inflammatory response of the HGF/c-Met pathway was also found in experimental autoimmune encephalomyelitis. Overall, it can be speculated that activation of c-Met in HRS cells might alter the immunosuppressive properties of the reactive cells by secretion of certain factors that shape the microenvironment in a way that
is less beneficial for survival and growth of the HRS cells.

In conclusion, despite its oncogenic function in CHL cell lines and other malignancies, c-Met expression by tumor cells in CHL patients predicts a very favorable outcome, independently of disease stage. Future studies should show whether c-Met can be used in clinical practice as a pre-treatment marker to select suitable patients for less toxic treatment regimens.

Authorship and Disclosures

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the IC MJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

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