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Myc protein overexpression is a feature of progression and adverse prognosis in multiple myeloma.

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Running Title: Myc expression in multiple myeloma
Abstract:

Objective: Prognostic and predictive markers in multiple myeloma are continuously explored because of the heterogeneity of the tumor biology. Myc protein is the final product from activating MYC oncogene but the prognostic impact in multiple myeloma is not well described.

Methods: In a population-based cohort of 194 untreated, newly diagnosed patients with multiple myeloma, we assessed myc protein expression using CD138/myc immunohistochemical double stain and collected clinicopathological data.

Results: Cases with myc protein expression ≥40% (myc\textsuperscript{HIGH}) had a median overall survival of 11 months compared to 48 months in cases of myc protein expression <40% (myc\textsuperscript{LOW}) (p<0.01). Myc\textsuperscript{HIGH} was significantly correlated to R-ISS, high proliferation index, high percentage of plasma cell in bone marrow, plasmablastic morphology, high calcium level and abnormal karyotype. In multivariate survival analyses, myc\textsuperscript{HIGH} was independently associated to inferior overall survival with a hazard ratio of 2.5.

Conclusion: Our results indicate myc protein overexpression to be associated with advanced multiple myeloma and poor prognosis.

Keywords: Multiple myeloma, hematopoiesis and hematopathology, molecular cytogenetics.

Introduction

Multiple myeloma (MM) is a disease of monoclonal plasma cells developing from monoclonal gammopathy of uncertain significance (MGUS) in a multi-step fashion accumulating genetic abnormalities (1). Symptomatic MM is characterised by end organ damage (CRAB-criteria) including hypercalcemia, renal impairment, anaemia and lytic bone disease (2). Prognostic and predictive markers are important due to striking differences in clinical outcome and response to treatment (1).
Well established prognostic markers are the International Staging System (ISS) and adverse cytogenetically abnormalities identified by fluorescence in situ hybridisation (FISH), the most important being t(4;14), t(14;16) and del17p. Adverse FISH, lactate dehydrogenase (LDH) in serum and ISS are integrated into a revised ISS (R-ISS) (3). However, even within these risk groups there is a substantial heterogeneity in prognosis. Revealing other prognostic markers is therefore pertinent.

MYC located on chromosome 8 is frequently deregulated in neoplastic cells and therefore an object of interest in many types of cancer. MYC is the driver oncogene of Burkitt lymphoma, e.g. t(8;14) (4) and myc activation at both genetic and protein level is associated with poor prognosis in diffuse large B-cell lymphoma, especially with concomitant activation of Bcl2 (double hit lymphoma) (5). The data for myc activation and prognosis in MM are mixed (6-11).

Myc protein binds to 10-15% of all promotor regions and is pivotal in cell cycle regulation, cell growth and metabolism. MYC is tightly controlled in normal cells, but as an activated oncogene contributes to malignant transformation by promoting uncontrolled cell proliferation, genomic instability and metabolic reprogramming in tumor microenvironment (12).

MYC activation is reported in up to 95% of multiple myeloma cell lines and 10-67% of MM tumors depending on the method used e.g. karyotyping, FISH or gene expression analysis (8, 13-15). MYC can be activated by amplification or translocation e.g. juxtaposing MYC at chromosome 8 with IgH at chromosome 14 or IgK/IgL at chromosome 2 and 22, respectively. Rearrangements of MYC in MM are often more complex though, involving translocation, inversion, insertion and deletion of frequently three chromosomes, making it a challenge to detect by FISH (9, 13, 16). Added to this, myc activation not only occurs on the genetic level, but also on other levels such as epigenetically, e.g. through inactivation of miRNA (17). Therefore, myc protein as the final product might be the most genuine way to detect MYC activation. Immunohistochemistry is a broadly available method, which makes myc protein expression easy to detect in daily clinical practice. Recent studies found myc protein expression in ≥ 1% of myeloma cells in 60-84% cases of MM using double immunohistochemically (IHC) staining with the plasma cell marker CD138 (8, 18). The prognostic
value of different myc protein levels has until now not been explored. The aim of this study was to
describe the clinicopathological correlations and prognostic impact of myc protein expression.

Patients and Methods

Patients

We analysed a population based cohort of 194 consecutive, untreated patients diagnosed with MM
between January 1st 2006 and June 30th 2010 in the Region of Southern Denmark as previously
reported (19). Clinical data was retrieved from the Danish Multiple Myeloma Registry and from
hospital medical records. One patient was excluded because of inadequate material left for myc
analysis. Treatment response was recorded according to IMWG 2006 criteria (20). The project was
approved by the Ethical Committees for the Region of Southern Denmark and was in accordance with
the current version of the Helsinki Declaration.

Tissue Samples, Processing and IHC

We used sections of two-micrometer from formalin-fixed paraffin-embedded bone marrow biopsy or
coagulum from aspirates. CD138/myc double stain was automated using the BenchMark Ultra
immunostainer (Ventana Medical Systems, Tucson, AZ) in a sequential set up. Epitope retrieval was
performed using Cell Conditioning Solution 1 (Ventana) for 64 min. at 99°C. In the first sequence,
myc staining was performed using clone EP121 (Y69), (Epitomics, Abcam, Cambridge, UK), diluted
1:100 and incubated 32 min at 36°C visualized by the OptiView-DAB detection system (Ventana).
The first sequence was completed by 4 minutes of denaturation at 94°C. In the second sequence
CD138 staining was performed using clone B-A38, (Ventana) with incubation time of 16 min at 36°C
and the UltraView-RED detection system (Ventana). Myc protein expression was assessed by
counting 200 plasma cells (100 cells in two different areas). Plasma cells were identified by a red
membrane staining of CD138. Myc was considered positive, when the nucleus stained distinct brown.
Weak brown was considered negative. Tumor burden, plasmablastic morphology and proliferation index by Ki-67/CD138 was assessed as already described (19).

Cytogenetics

Karyotyping was carried out in 181 patients and FISH with positive plasma cell identification in 192 patients as previously described (21, 22) using the following commercial probes: Kreatech™ 1q21/SRD (1p36) Specific FISH probe (Kreatech Biotechnology, Leica Biosystems, Amsterdam, The Netherlands), LSI 13q and LSI 17p, 14q32 dual-color break-apart probe, t(4;14), t(11;14) and t(14;16) dual-color fusion probes (Abbott Laboratories, Abbott Park, Illinois). One hundred nuclei of clonal plasma cells were counted and cut off for positive findings set at 10%.

Statistical methods

Categorical data were compared using Fisher’s exact test and two-sided p-value. P-values <0.05 were considered significant. One patient with unavailable medical records, twenty-two with continuously smoldering MM (SMM) during follow-up and five patients treated with dexamethasone or bisphosphonate only were excluded from survival analyses. Kaplan-Meier was used to estimate overall survival (OS) defined as time from diagnosis to last follow-up or death. Multivariate analyzes were calculated using Cox Regression analyses of parameters of prognostic importance in univariate analysis. The statistical analyses were performed with the SPSS version 21 software (IBM, Armonk, New York).

Results

The cohort had a median age of 71 years (43-93 years). Clinical and pathological data were described previously (19) and are summarized in Table 1. Myc protein was expressed in 177 cases (92%) with a
range of 1-65% myc-positive plasma cells (Figure 1). The distribution is shown in Figure 2. Twenty-nine patients (15%) had SMM at time of diagnosis. Among these, only two (7%) showed ≥10% myc-positive plasma cells compared to 72/164 (44%) among symptomatic cases (p≤0.01). Seven SMM patients later progressed to MM with a median time of 23 months, one with myc expression ≥10% and six with <10% myc-positive plasma cells.

Median follow up in 165 patients receiving anti myeloma treatment was 45 months. Fifty-one (31%) received high-dose chemotherapy with high dose melphalan 200 mg/m² and autologous stem cell transplantation (ASCT), 114 (69%) were given non-intensive alkylator based triplets including novel drugs, thalidomide or bortezomib (67%), and a small percentage got only a doublet with lenalidomide, thalidomide or bortezomib plus dexamethason (2%). Fifty-nine (36%) of 163 patients achieved at least very good partial response (VGPR). Two patients died before response evaluation was possible.

Initial survival analysis of myc protein expression was done with myc stratified in ten percent intervals. This showed no significant difference in OS between myc expression intervals below 40%, however OS was significantly inferior if myc protein expression was ≥40% (p<0.01) (Figure 3A). Median OS was only 11 months for cases with myc ≥40% (myc\textsuperscript{HIGH}) compared to 48 months for cases <40% (myc\textsuperscript{LOW}) (p<0.01) (Figure 3B)). Univariate survival analyses also showed significantly inferior OS with increasing R-ISS score (p<0.01), age>65 years (p<0.01), high LDH level (p<0.01), proliferation index >9% (p<0.01) and non-intensive treatment (no-ASCT vs ASCT) (p<0.01) (data not shown).

Myc\textsuperscript{HIGH} was significantly correlated to R-ISS, high proliferation index, high percentage of plasma cell in bone marrow, plasmablastic morphology, high calcium level, abnormal karyotype and to female gender (Table 1). There was a trend toward inferior response to treatment since only two cases of myc\textsuperscript{HIGH} (13%) achieved at least VGPR compared to 57 of 148 (39%) cases of myc\textsuperscript{LOW} (P=0.05). Myc\textsuperscript{HIGH} was not correlated to high LDH level, low haemoglobin or high creatinine (Table 1). Our cohort contained too few cases with FISH defined high risk cytogenetics to allow correlative analysis.
In multivariate Cox regression survival analysis incorporating age, high risk FISH, intensive treatment (+/- ASCT) and ISS, myc^{HIGH} was independently associated with inferior OS with a hazard ratio of 2.5 (Table 2). In this model age lost prognostic significance. Data incorporating R-ISS instead of ISS also showed significant inferior OS in cases of myc^{HIGH} with a hazard ratio of 2.0 in spite of data being available in only 124 cases due to incomplete data (data not shown).

**Discussion**

Detecting rearrangements of MYC in MM by FISH requires multiple probe sets and is a complex and costly method for daily clinical practice. Activation of MYC can be estimated by gene expression assays detecting selected MYC related genes, mRNA or DNA sequence capture technique but these methods are not widely available. Instead, we applied a commercial antibody for immunohistochemical identification of the myc protein, and this method is reliably, easy to perform and less costly. It is difficult to assess the myc protein expression in percentage of plasma cells when the tumor burden is low and the infiltration pattern diffuse. To overcome this we used immunohistochemical double staining with the plasma cell marker CD138 and myc.

We found varying myc protein expression in 92% of 193 newly diagnosed MM with a range of 1-65% positive plasma cells using the immunohistochemical double staining approach. Other authors have reported myc protein expression in 84% of 26 newly diagnosed MM (range 1-90%) (18) and 60% in 48 patients (36 newly diagnosed and 12 relapsed) (8). The same myc clone Y69 was used in these studies, but a different clone for CD138 (Dako clone MI15). Patient cohorts were different from our study. Patients were younger (median age 65 years) and 81% were treated with upfront ASCT (18) compared to only 31% treated with ASCT and a median age of 71 years in our cohort. Different thresholds of myc positivity might also influence and cause small differences since weak nuclear staining is considered negative.
We found myc^{HIGH} correlated to high proliferation index, high tumor burden and plasmablastic morphology, high calcium level, high β2-microglobulin level (data not shown), R-ISS3 and a trend towards correlation with inferior response to treatment (Table 1). Other groups also found correlation of myc protein to high calcium and extramedullary disease (11), high proliferation index by Ki-67 (18), and increased expression of myc related genes in gene expression (GEP) analyses correlated to high plasma cell labelling index and high ISS score (8). FISH studies showed high β2-microglobulin levels correlated to MYC rearrangements (14). In spite of the reported association between myc expression and aggressive disease phenotype we did not observe myc^{HIGH} to be more frequent in cases with high risk FISH and this observation is also in line with results found by others (9, 14). However, in our patients myc^{HIGH} was clearly related to abnormal karyotype.

Recent studies have shown gain of myc protein expression when transforming from MGUS into plasma cell myeloma (18) indicating myc activation to be a possible early secondary event as well as a late event. We found significantly lower expression of myc in SMM compared to MM, and one of two patients with SMM and myc protein expression >10% progressed to MM during follow up, but so did six patients with SMM and myc protein expression <10%. These data indicate MYC as a possible gene among others involved in progression.

Primary cytogenetic pathways in MGUS/MM are hyperdiploid (HRD) or non-hyperdiploid (NHRD) pathway, the latter characterized by the recurrent translocations t(4;14), t(6;14), t(11;14), t(14;16) and t(14;20) (1). MYC rearrangements are shown to be evenly distributed in HRD and NHRD tumors (23), rare or absent in MGUS and SMM (16), associated with advanced tumors and therefore considered to be a late secondary genetic event in MM (13, 15). MM cell lines are mostly generated from advanced extramedullary tumors that are able to proliferate independently of the microenvironment in bone marrow, which is in contrast to most human myeloma cells (13).
The increased myc activity in these cell line tumors also indicate myc activation to be associated with advanced MM. Our findings of myc protein overexpression correlating with abnormal karyotype support this hypothesis of myc contributing to myeloma growth independent of microenvironment, since detection of abnormal karyotype is conditional on the ability of plasma cells to divide in vitro.

Thus, myc protein expression >40% seems to reflect the presence of a dominant clone with high proliferative capacity and with potentials of extramedullary spread, and myc\(^{HIGH}\) was in our cohort associated with a very poor prognosis with a median OS below 12 months. In our cohort myc\(^{HIGH}\) identified 9% of newly diagnosed patients with an extra high risk disease. Myc\(^{HIGH}\) was observed in all ISS subgroups. Thus, myc\(^{HIGH}\) candidates to be further tested for and potentially integrated into a new revised ISS.

Reports on myc translocation and prognosis are equivocal. Intergroupe Francophone du Myélome (IFM) found no impact on FISH detected MYC rearrangements on prognosis in 571 symptomatic, newly diagnosed MM patients, but the authors concluded that MYC is activated by other mechanisms than translocations, which might have hidden the prognostic impact (6). This is supported by a recent study finding no correlation between myc protein expression and MYC translocation (11). Other authors found significantly inferior progression free survival (PFS) and OS with FISH detected MYC rearrangements in 101 patients with advanced disease (7), and in 55 patients where MYC rearrangements were detected by a combined FISH and DNA sequence technique (9). Absence of MYC translocation was a positive prognostic factor in OS multivariate analyses incorporating intensive treatment and absence of adverse translocations (HR 4.1, CI 1.1-14.8).

Previous small case studies indicated inferior PFS or OS associated to myc protein expression in univariate analyses (8, 11, 18). These studies used different cut-offs of myc protein expression (from 1% to 30%) and one study did not use double staining of myc protein and CD138 complicating assessment of cases with small tumor burden, leading to exclusion of these cases (11).
In conclusion, we found myc protein expression to be of clinical and prognostic importance in a population-based cohort of 165 newly diagnosed patients with MM. Myc protein expression $\geq 40\%$ was significantly associated with inferior OS ($p<0.01$) and in multivariate survival analyses myc$^\text{HIGH}$ was independently associated to inferior OS with a hazard ratio of 2.5. Our results are based on retrospective analyses in a heterogeneous treated cohort of patients and should be confirmed in a prospective randomized trial of equally treated newly diagnosed patients. Ongoing studies on myc inhibition as a potential target of treatment (24) support the importance on studying this subject further.

Conflict of Interest

The authors declare no conflict of interest.

References


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Table 1. Clinicopathological characteristics correlated to myc expression. Myc protein ≥40% was significantly correlated to R-ISS, high proliferation index, high percentage of plasma cell in bone marrow, plasmablastic morphology, high calcium level, abnormal karyotype and there was a trend toward inferior response to treatment.

<table>
<thead>
<tr>
<th>Characteristics (n)</th>
<th>Myc &lt; 40% (n=150)</th>
<th>Myc ≥ 40% (n=15)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age ≥ 65 y (165)</td>
<td>104 (69%)</td>
<td>9 (60%)</td>
<td>0.56</td>
</tr>
<tr>
<td>Female (165)</td>
<td>74 (49%)</td>
<td>12 (80%)</td>
<td>0.02</td>
</tr>
<tr>
<td>Low Hgb (Hgb&lt;7mmol/l) (165)</td>
<td>119 (79%)</td>
<td>14 (93%)</td>
<td>0.31</td>
</tr>
<tr>
<td>High creatinine (Cr&gt;130µmol/l) (165)</td>
<td>44 (29%)</td>
<td>5 (33%)</td>
<td>0.77</td>
</tr>
<tr>
<td>High calcium (Ca-ionized&gt;1,35 mmol/l) (163)</td>
<td>45 (30%)</td>
<td>10 (67%)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>LDH above normal (161)</td>
<td>17 (12%)</td>
<td>4 (27%)</td>
<td>0.11</td>
</tr>
<tr>
<td>ISS score (151)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>53 (39%)</td>
<td>2 (14%)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>39 (28%)</td>
<td>3 (21%)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>45 (33%)</td>
<td>9 (64%)</td>
<td>0.06</td>
</tr>
<tr>
<td>R-ISS score (124)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>32 (28%)</td>
<td>1 (10%)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>71 (62%)</td>
<td>5 (50%)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>11 (10%)</td>
<td>4 (40%)</td>
<td>0.02</td>
</tr>
<tr>
<td>Severe bone disease § (164)</td>
<td>92 (62%)</td>
<td>12 (80%)</td>
<td>0.16</td>
</tr>
</tbody>
</table>
Morphology of plasma cells (165)

<table>
<thead>
<tr>
<th>Morphology</th>
<th>Count (Percentage)</th>
<th>Reference</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasmablastic</td>
<td>50 (33%)</td>
<td>165</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>% plasma cells in BM (165)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤ 20%</td>
<td>32 (21%)</td>
<td>165</td>
<td></td>
</tr>
<tr>
<td>&gt; 20% and ≤ 50%</td>
<td>47 (31%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>&gt; 50% and ≤ 75%</td>
<td>37 (25%)</td>
<td>165</td>
<td></td>
</tr>
<tr>
<td>&gt; 75%</td>
<td>34 (23%)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Proliferation Index (by Ki-67/CD138) (165)

<table>
<thead>
<tr>
<th>Index</th>
<th>Count (Percentage)</th>
<th>Reference</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>≤ 1%</td>
<td>32 (21%)</td>
<td>165</td>
<td></td>
</tr>
<tr>
<td>&gt;1 and ≤ 4%</td>
<td>47 (31%)</td>
<td>165</td>
<td></td>
</tr>
<tr>
<td>&gt;4 % and ≤ 9%</td>
<td>37 (25%)</td>
<td>165</td>
<td></td>
</tr>
<tr>
<td>&gt; 9%</td>
<td>33 (22%)</td>
<td>165</td>
<td></td>
</tr>
</tbody>
</table>

Adverse cytogenetic, FISH (164)

<table>
<thead>
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<th>Cytogenetic</th>
<th>Count (Percentage)</th>
<th>Reference</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>del(17p)</td>
<td>9 (6%)</td>
<td>161</td>
<td></td>
</tr>
<tr>
<td>t(4;14)</td>
<td>10 (7%)</td>
<td>155</td>
<td></td>
</tr>
<tr>
<td>t(14;16)</td>
<td>4 (4%)</td>
<td>112</td>
<td></td>
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</tbody>
</table>

Abnormal karyotype (154)

<table>
<thead>
<tr>
<th>Karyotype</th>
<th>Count (Percentage)</th>
<th>Reference</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 (11%)</td>
<td>15 (11%)</td>
<td>164</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ASCT (165)</td>
<td>47 (31%)</td>
<td>165</td>
<td>0.48</td>
</tr>
<tr>
<td>Bortezomib</td>
<td>33 (22%)</td>
<td>165</td>
<td>0.31</td>
</tr>
<tr>
<td>Thalidomide</td>
<td>28 (19%)</td>
<td>165</td>
<td>0.46</td>
</tr>
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</table>

Treatment response ≥VGPR (163)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Count (Percentage)</th>
<th>Reference</th>
<th>p-value</th>
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</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>57 (39%)</td>
<td>163</td>
<td>0.05</td>
</tr>
</tbody>
</table>

§ Multiple lytic regions or fracture
NC = not calculable
Table 2. Cox proportional multivariate analysis of prognostic markers of significance in univariate analysis showing myc protein expression ≥40% as a single adverse prognostic factor with a hazard ratio of 2.5 (p<0.01).

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myc ≥40%</td>
<td>2.5</td>
<td>1.39-4.59</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>No ASCT</td>
<td>2.8</td>
<td>1.74-4.57</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ISS</td>
<td>1.6</td>
<td>1.23-1.99</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Adverse FISH</td>
<td></td>
<td></td>
<td>0.78</td>
</tr>
<tr>
<td>Age &gt;65</td>
<td></td>
<td></td>
<td>0.53</td>
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