

## ORIGINAL ARTICLE

## Proliferation, apoptosis, and intratumoral vascularity in multiple myeloma: correlation with the clinical stage and cytological grade

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**Aims:** Abnormalities involving proliferation, apoptosis, and angiogenesis are important in tumorigenesis. The purpose of this study was to examine these three biological processes, and their relation with the clinical stage and cytological grade in multiple myeloma (MM).

**Methods:** Fifty four newly diagnosed patients with MM were studied by immunohistochemistry using bone marrow clot sections. Proliferation and apoptosis were evaluated for the proportion of MM cells (indicated by morphology and CD138 reactivity) positive for the Ki67 antigen and single stranded DNA (ssDNA), respectively. Angiogenesis was evaluated by measuring the intratumoral microvessel density (IMVD) and by assessing the immunoreactivity of vascular endothelial growth factor (VEGF).

**Results:** There were 30 men and 24 women (median age, 65 years; range, 37–84). At initial presentation, 15 (28%) were in Durie stage I, 15 (28%) in stage II, and 24 (44%) in stage III. Advanced clinical stage correlated with high cytological grade ( $p < 0.03$ ). The medians for Ki67, ssDNA, and IMVD were 4.4% (range, 0–15%), 0.2% (range, 0–2.8%), and 15.5 (range, 0–63), respectively. Among these three continuous parameters, the only significant correlation was that between Ki67 and IMVD ( $p < 0.0001$ ). Both Ki67 and IMVD also correlated with the clinical stage, cytological grade, and VEGF positivity ( $p < 0.05$ ). No correlation was found between ssDNA and all of the other parameters.

**Conclusions:** These data suggest that proliferation is associated with angiogenesis in MM. Furthermore, proliferation and angiogenesis, but not apoptosis, may be important in disease progression. Lastly, increased production of VEGF may be one of the contributing factors to the increase in intratumoral vascularity seen in advanced MM.

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Multiple myeloma (MM) is a progressively fatal disease characterised by the accumulation of malignant plasma cells in the bone marrow.<sup>1</sup> Previous studies have provided insights into the pathogenesis of this disease, and defects involving proliferation, apoptosis, and angiogenesis are believed to be important.<sup>2</sup> Proliferation as measured by bromodeoxyuridine labelling of MM cells has been shown to be an important prognostic factor for MM.<sup>3</sup> Proliferation assessed by immunohistochemistry to detect the Ki67 antigen is also associated with advanced pathological stage of MM.<sup>4</sup> More recent studies of MM have examined the roles of angiogenesis and apoptosis in this disease. Abnormalities of the apoptosis related proteins have been observed in MM. For instance, overexpression of bcl-2 is present in most patients with MM,<sup>5</sup> and this abnormality may mediate the resistance of MM cells to apoptosis induced by dexamethasone and interleukin 6 (IL-6) deprivation.<sup>6,7</sup> In addition, mutations of the tumour suppressor gene p53 are present in 5% of patients with MM and 20–40% of patients with acute plasma cell leukaemia. As for angiogenesis, a recent study showed that an increase in the bone marrow microvessel density in MM is associated with a worse prognosis.<sup>8</sup>

"Proliferation as measured by bromodeoxyuridine labelling of multiple myeloma cells has been shown to be an important prognostic factor for multiple myeloma"

To our knowledge, no reported studies have evaluated proliferation, apoptosis, and angiogenesis at the same time. Vacca *et al* reported an association between increased intratumoral vascularity and a high plasma cell labelling index.<sup>9</sup> Nevertheless, how apoptosis is related to proliferation or

intratumoral vascularity is unclear. In addition, little is known about the relation between these biological processes and the clinical stage or cytological grade of MM. Thus, the purpose of our study was to examine proliferation, apoptosis, and angiogenesis in 54 patients with newly diagnosed MM. Specifically, we evaluated whether there is a correlation among these three biological processes, and how these processes are related to the clinical stage or cytological grade.

## METHODS

## Patient selection

Fifty four newly diagnosed patients with MM were randomly identified and retrieved from the file between 1988 and 2000 in the department of laboratory medicine, Nagoya University School of Medicine.

## Monoclonal antibodies and immunohistochemistry

Monoclonal antibodies used in our study were directed against the following antigens: Ki67, (1/100 dilution; Immunotech, Marseille, France), CD34 (1/100 dilution; Immunotech), single stranded DNA (ssDNA) (1/100 dilution; Dako, Kyoto, Japan), and vascular endothelial growth factor (VEGF; 1/200 dilution; Santa Cruz Biotechnology, Santa Cruz, California, USA). Immunohistochemistry was performed on 3 µm tissue sections of formalin fixed, paraffin wax embedded bone

**Abbreviations:** IL, interleukin; IMVD, intratumoral microvessel density; MM, multiple myeloma; ssDNA, single stranded DNA; VEGF, vascular endothelial growth factor

marrow aspirate clots. After dewaxing in xylene and dehydration through graded concentrations of ethanol, the tissue sections were subjected to microwave antigen retrieval (750 W; citrate buffer, 0.01 mol/litre, pH 6.0) for five minutes. The tissue sections were subsequently put into an automated immunostainer (Ventana Medical System, Tucson, Arizona, USA). For each case, double marker analysis was performed combining CD138 with Ki67 and ssDNA. Reactivity for Ki67 and ssDNA was detected by a streptavidin-alkaline phosphatase system with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphatase as the chromogen. After washing in phosphate buffered saline (0.1 mol/litre, pH 7.5) three times, immunohistochemical staining for CD138 (1/40 dilution; Serotec, Kidlington, UK) was performed using a streptavidin-biotin-peroxidase linking system and diaminobenzidine as the chromogen. Counterstaining was then performed using haematoxylin.

#### Evaluation of immunoreactivity

Two thousand CD138 positive MM cells from a minimum of five representative areas were evaluated microscopically under  $\times 400$  magnification. The reactivity for Ki67 and ssDNA is expressed as a percentage of CD138 positive MM cells positive for these two markers; the staining intensity of Ki67 and ssDNA was irrelevant in the scoring. The assessment of VEGF staining was based on the sum of the points scored for the VEGF staining intensity (0, negative; 1, slight staining; 2, moderate staining; 3, strong staining) and the points scored for the percentage of MM cells positive for VEGF (0, no positive cells; 1, 1–25% positive cells; 2, 26–50% positive cells; 3, > 50% positive cells). A final score of > 2 points was consid-

ered to be VEGF positive, whereas a final score of  $\leq 2$  points was considered to be VEGF negative.<sup>10</sup>

Intratumoral vascularity was assessed using the intratumoral microvessel density (IMVD), which was based on a modified method described previously.<sup>11</sup> Briefly, the five most vascular areas were selected under a microscope using low power scanning. Vessels highlighted by CD34 immunoreactivity were counted under light microscopy with  $\times 400$  magnification. IMVD was the average of the number of vessels derived from the five areas.

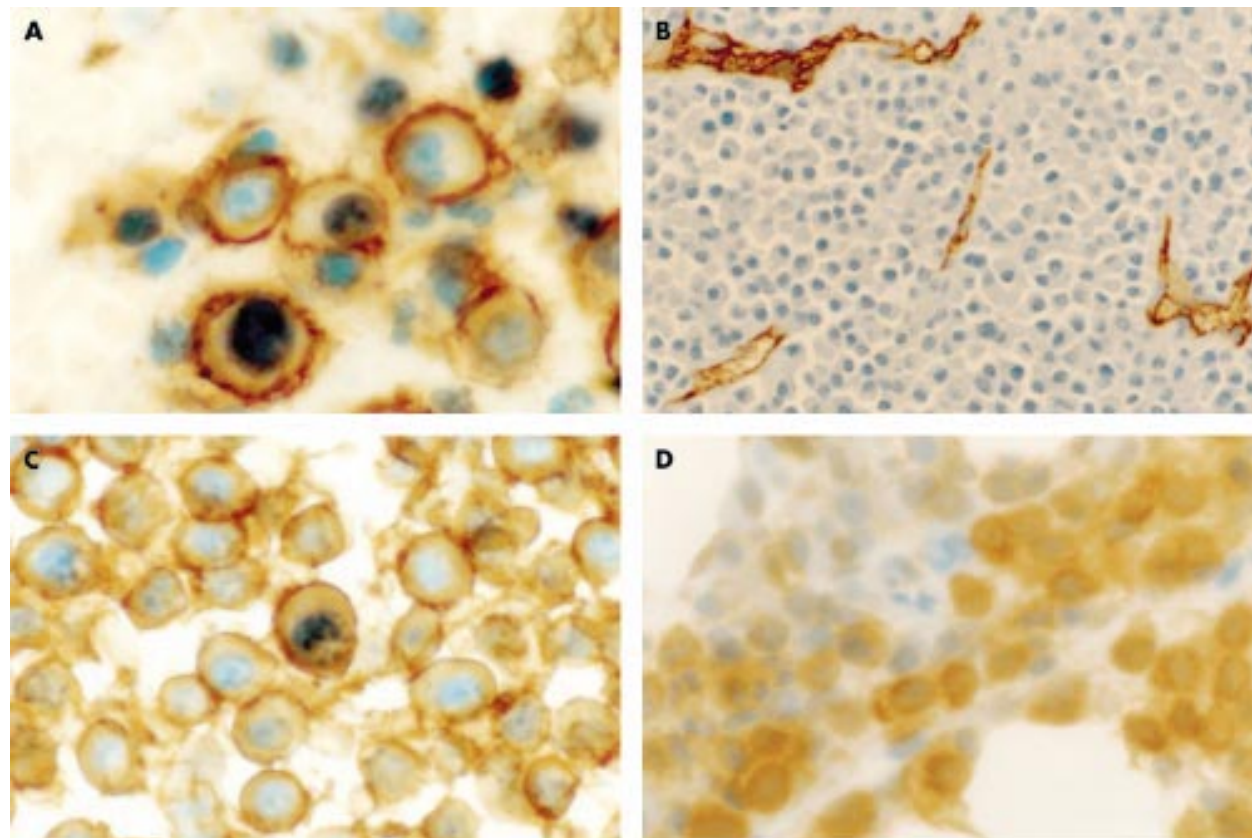
#### Statistical analysis

The Mann-Whitney U test was used to determine the significance of the differences in Ki67, IMVD, and ssDNA among the three clinical stages (Durie's) and the three cytological grades (mature, immature, blastic). Pairs of numerical variables including Ki67, ssDNA, and IMVD were analysed using the Spearman correlation. Correlation between the categorical (clinical stage, pathological stage, and VEGF) and continuous variables (Ki67, ssDNA, and IMVD) was done by the Kruskal-Wallis method and the Cox hazard proportional model. A p value of < 0.05 was considered to be significant. The Wilcoxon rank sum test was used to determine whether there was a significant difference between Ki67 or IMVD in VEGF positive versus VEGF negative MM.

## RESULTS

#### Patients, clinical stage, and cytological grade

There were 30 men and 24 women (median age, 65 years; range, 37–84). At initial presentation, 15 (28%) were in Durie



**Figure 1** (A) Ki67 immunoreactivity (blue nuclear staining) in CD138 positive (brown cell surface staining) cells in a bone marrow clot section from a patient with multiple myeloma (original magnification,  $\times 1000$ ). (B) measurement of intratumoral vascular density using CD34 immunostaining to highlight intratumoral blood vessels (original magnification,  $\times 200$ ). (C) Single stranded DNA immunostaining (blue nuclear staining) in CD138 positive (brown cell surface staining) cells in a bone marrow clot section from a patient with multiple myeloma (original magnification,  $\times 1000$ ). (D) Immunostaining of vascular endothelial growth factor in a patient with multiple myeloma (original magnification,  $\times 400$ ).

**Table 1** Correlation between clinical stage and proliferation index, apoptosis index, and intratumour microvessel density in 54 multiple myelomas

	Stage I (N=15) Mean (SD) (%)	Stage II (N=15) Mean (SD) (%)	Stage III (N=24) Mean (SD) (%)	p Value
Ki67	0.72 (0.88)	3.72 (3.94)	7.15 (3.70)	<0.0001
Intratumour microvessel density	5.29 (4.38)	14.47 (8.07)	22.20 (14.15)	<0.0001
Single stranded DNA	0.05 (0.12)	0.17 (0.25)	0.24 (0.60)	>0.05

**Table 2** Correlation between Bartl's pathological stage and proliferation index, apoptosis index, and intratumour microvessel density in 54 multiple myelomas

	Plasmablast (N=13) Mean (SD) (%)	Immature (N=21) Mean (SD) (%)	Mature (N=20) Mean (SD) (%)	p Value
Ki67	7.28 (4.00)	4.64 (3.80)	1.37 (1.54)	<0.001
Intratumour microvessel density	23.39 (17.96)	15.10 (9.91)	9.56 (7.76)	<0.05
Single stranded DNA	0.12 (0.19)	0.23 (0.60)	0.25 (0.64)	>0.05

stage I, 15 (28%) in stage II, and 24 (44%) in stage III. According to Bartl's pathological stage, there were 13 (24%) in plasmablastic cell type, 21 (39%) in immature cell type, and 20 (37%) in mature cell type. Plasmablastic was considered to be high grade. Advanced stage disease correlated with high cytological grade ( $p < 0.05$ ).

#### Expression of the Ki67 antigen

An illustration of Ki67 immunostaining is shown in fig 1A, and the results are summarised in table 1. The proportion of Ki67 positive myeloma cells was variable from case to case, ranging from 0% to 15%, with an overall median of 4.4%. Ki67 labelled MM cells increased with advanced clinical stage. As summarised in table 1, the mean percentages of Ki67 positive MM cells in Durie's clinical stage I, II, and III were 0.72%, 3.72%, and 7.15%, respectively. This positive correlation between Ki67 and clinical stage is significant ( $p < 0.0001$ ; Mann-Whitney U test). Similarly, the mean percentages of Ki67 positive MM cells increased with the cytological grade: 1.37% in the mature cell type, 4.64% in the immature cell type, and 7.28% in the plasmablastic cell type. This correlation was significant ( $p < 0.001$ , Mann-Whitney U test; table 2).

#### IMVD in MM

Figure 1B shows typical results for IMVD. The median IMVD was 5.29 vessels/high power field in stage I, 14.47 vessels/high power field in stage II, and 22.20 vessels/high power field in stage III (table 1). This correlation was significant ( $p < 0.0001$ ; Mann-Whitney U test). IMVD also increased with the cytological grade: 9.56 vessels/high power field in the mature cell type, 15.1 vessels/high power field in the immature cell type, and 23.39 vessels/high power field in the plasmablastic cell type. This correlation was also significant ( $p < 0.05$ ; Mann-Whitney U test; table 2).

#### Expression of ssDNA

The results of ssDNA are summarised in table 1, and illustrated in fig 1C. The percentages of ssDNA positive MM cells were less variable than those of Ki67 or IMVD, ranging from 0% to 2.8%, with a median of 0.2%. As shown in table 1 and table 2, there was no significant correlation between the percentage of ssDNA positive cells and the clinical stage or cytological grade ( $p > 0.05$ ; Mann-Whitney U test).

#### Expression of VEGF

To evaluate whether IMVD is related to VEGF values, the expression of VEGF was assessed in all 54 cases. The immunostaining for VEGF is shown in fig 1D. Six cases were VEGF

negative and 48 cases were VEGF positive. There was no significant correlation between VEGF positivity and either the clinical stage or the cytological grade.

#### Correlation between Ki67, IMVD, VEGF, and ssDNA

Ki67 correlated with IMVD ( $p < 0.05$ ; Spearman). Using the Wilcoxon rank sum test, we found that Ki67 was also significantly higher in the VEGF positive than the VEGF negative cases (4.67% v 2.40%;  $p < 0.001$ ). Similarly, IMVD was also significantly higher in VEGF positive than VEGF negative cases (16.23 v 10;  $p < 0.001$ ). There was no significant correlation identified between ssDNA and the other three parameters.

#### DISCUSSION

In summary, we identified a significant correlation between Ki67 and IMVD, and between these two continuous variables with the clinical stage, cytological grade, and VEGF positivity. Thus, patients with MM who have a high clinical stage/cytological grade tend to have a higher rate of proliferation, higher intratumoural vascularity, and increased VEGF in the neoplastic cells. In contrast, apoptosis as assessed by ssDNA labelling appears to be an independent parameter.

With regard to the correlation between Ki67 and the clinical stage, our findings are in keeping with those of an earlier study,<sup>12</sup> although another study that examined Ki67 by immunohistochemistry applied to bone marrow biopsy specimens showed no significant correlation between Ki67 and the clinical stage.<sup>13</sup> Although this discrepancy may be related to the use of different staining techniques, it is also possible that the difference may be related to the inclusion of non-myeloma cells during the measurement of Ki67 labelling. This may occur because erythroid precursors can be morphologically confused with MM cells in bone marrow biopsy sections. Because erythroid precursors have the highest proliferative activity in the bone marrow,<sup>14</sup> erroneous inclusion of these cells during the estimation of Ki67 will lead to falsely high results. To avoid this potential problem, we used CD138 immunostaining to enhance our specificity during Ki67 staining.

The observation that Ki67 correlates with IMVD is interesting. Vacca *et al* demonstrated this correlation in MM previously. To explain the underlying mechanism for this correlation, the authors have suggested that proliferating MM cells may promote angiogenesis through autocrine and paracrine release of IL-6, a potent angiogenic factor.<sup>9</sup>

The increase in IMVD in MM of advanced clinical stage may also be related to the release of additional angiogenic

cytokines, such as IL-8 and granulocyte-macrophage colony stimulating factor, by the host bone marrow cells. In addition, as shown in our study, increased production of VEGF by MM cells may contribute to the increased intratumoral vascularity seen in advanced stages. Similar to our findings, others have found a correlation between angiogenesis and VEGF expression in some types of solid tumours<sup>15 16</sup> and acute myeloid leukaemia.<sup>17</sup> Bellamy *et al* also showed that VEGF is overexpressed by MM cells, but not by benign plasma cells from normal bone marrow.<sup>18</sup> Similarly, Dankbar *et al* demonstrated the expression of VEGF by myeloma cell lines and MM patient samples. Because the receptors for VEGF (Flt-1 and KDR) are expressed in normal myeloid and monocyte cells,<sup>19</sup> it is possible that VEGF plays a role in the angiogenesis and growth of MM through a paracrine or autocrine mechanism. Taken together, the correlation between the expression of VEGF in MM cells and IMVD in our study suggests that VEGF may directly contribute to the angiogenesis of MM through an autocrine pathway.

"Patients with multiple myeloma who have a high clinical stage/cytological grade tend to have a higher rate of proliferation, higher intratumoral vascularity, and increased vascular endothelial growth factor in the neoplastic cells"

Although we found a correlation between angiogenesis and proliferation in MM, this correlation is not seen uniformly in solid tumours. For instance, IMVD does not correlate with tumour cell proliferation in epidermoid lung carcinoma,<sup>10</sup> breast ductal carcinoma,<sup>20 21</sup> and carcinomas of the oesophagus.<sup>22</sup> In contrast, Vermeulen *et al* found an association between Ki67 labelling and intratumour vascularity in colorectal adenocarcinomas.<sup>16</sup> Thus, the relation between the IMVD and proliferation of tumour cells is probably specific to the tumour type. Furthermore, the mechanisms underlying the correlation between IMVD and proliferation seen in MM and some of the solid tumours may also be different.

Apoptosis is an important factor in tumorigenesis,<sup>23</sup> and previous studies have shown that spontaneous apoptosis occurs more frequently in tumours of high cytological grade and advanced clinical stage of disease.<sup>24 25</sup> Defects in apoptosis have been documented in MM.<sup>26</sup> Nevertheless, no comprehensive study has been performed to evaluate apoptotic activity in untreated MM. The rate of apoptosis is relatively low in untreated MM when compared with highly proliferative haematological disorders (such as acute leukaemia), but it is comparable to low grade haematological disorders (such as chronic lymphocytic leukaemia).<sup>27</sup> Importantly, there was no significant correlation between apoptosis and clinical stage, Ki67 labelling, or ssDNA labelling. Its independence from clinical stage may be related to the fact that bcl-2 overexpression also does not correlate with clinical stage. It is possible that defects in apoptosis in MM occur early in its pathogenesis and persist through the course of the disease, and may not be important in disease progression.

To conclude, our data suggest that proliferation is associated with angiogenesis in MM. Furthermore, proliferation and angiogenesis, but not apoptosis, may be important in disease progression. Lastly, increased production of VEGF may be one of the contributing factors to the increase in intratumoral vascularity seen in advanced MM.

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#### Take home messages

- In multiple myeloma (MM) proliferation appears to be associated with angiogenesis
- Proliferation and angiogenesis may be important in disease progression in MM
- However, apoptosis as assessed by ssDNA labelling appears to be an independent parameter
- Increased production of vascular endothelial growth factor may be a contributing factor to the increase in intratumoral vascularity seen in advanced MM

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## HISTORICAL PERSPECTIVES.....

### Lamashtu, “she who erases”, touched her stomach seven times to kill the child

Over the centuries, pregnancy has appeared to be a dangerous and often even lethal period of life, with many diseases threatening both mother and child. In modern times these diseases are often well recognised and may be adequately treated, with sufficient medical expertise and the appropriate political situation.<sup>1</sup> In ancient Mesopotamia, the source of pregnancy and childbirth associated pathology was sought in demonology. One specific demon, in particular, was held responsible for diseases of this sort, namely, Lamashtu (Akkadian): “she who erases”.<sup>2</sup>

Daughter of Anu, one of the greater gods, her appearance was as terrible as her work. Equipped with a hairy human body, the head of a lioness, teeth and ears of a donkey, and bird feet with sharp talons, she is often shown standing or kneeling on a donkey, nursing a pig and dog, and holding snakes. Her work included poisoning water with disease, killing plants, bringing nightmares, and causing tetanus in addition to persistent fevers.<sup>2</sup>

However, Lamashtu’s principle victims were unborn. Slipping into the house of a pregnant woman, she tried to touch the woman’s stomach seven times to kill the child. She would poison newborns by abducting the child from its wet nurse and feeding it with its own toxic milk. Mothers could also be killed, and she sometimes ate the flesh and drank the blood of adult men,<sup>2</sup> although it is not stated whether they were the fathers or just randomly picked individuals.

Recently, the long term mortality of mothers and fathers after pre-eclampsia was studied in a population based cohort.<sup>3</sup> Although women with pre-eclampsia had a higher long term risk of death, the survival of fathers involved in pre-eclampsia complicated pregnancies was not shortened in any way compared with fathers not involved in this kind of pregnancy.<sup>3</sup> This might be an illustration of Lamashtu specifically attacking the mothers of her young victims, with her adult male victims being random men crossing her path at the wrong time, in the wrong place.

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## Lamashtu, "she who erases", touched her stomach seven times to kill the child

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