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A Molecular Diagnostic Approach Able to Detect the Recurrent Genetic Prognostic Factors Typical of Presenting Myeloma

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Risk stratification in myeloma requires an accurate assessment of the presence of a range of molecular abnormalities including the differing IGH translocations and the recurrent copy number abnormalities that can impact clinical behavior. Currently, interphase fluorescence in situ hybridization is used to detect these abnormalities. High failure rates, slow turnaround, cost, and labor intensiveness make it difficult and expensive to use in routine clinical practice. Multiplex ligation-dependent probe amplification (MLPA), a molecular approach based on a multiplex polymerase chain reaction method, offers an alternative for the assessment of copy number changes present in the myeloma genome. Here, we provide evidence showing that MLPA is a powerful tool for the efficient detection of copy number abnormalities and when combined with expression assays, MLPA can detect all of the prognostically relevant molecular events which characterize presenting myeloma. This approach opens the way for a molecular diagnostic strategy that is efficient, high throughput, and cost effective.

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the study of tumors with low proliferation indices, such as myeloma, and yield results in only 35% of all patients in clinical trial samples. In contrast to metaphase cytogenetics, interphase fluorescence in situ hybridization (iFISH) performed on CD138-selected tumor cells offers a more viable approach, giving conclusive results for t(4;14), del(17p), and del(1p32)/gain(1q21), when enough cells are available and in up to 70% of trial patients (Myeloma IX trial, unpublished data). Nevertheless, this method is labor intensive and, as each lesion is assessed independently, the full panel of tests required to provide prognostically relevant information is both expensive and labor intensive. There is, therefore, a need for a novel diagnostic tool able to detect the prognostically relevant abnormalities in myeloma which is both high throughput and applicable in a clinical setting. Molecular approaches performed on DNA and RNA from CD138-selected PCs would fulfill these criteria and could be particularly useful in the clinic. Detecting chromosomal translocations by the overexpression of partner oncogenes using quantitative reverse transcription polymerase chain reaction (qRT-PCR) has been described previously, but for prognostic purposes the copy number variables also need to be assessed (Kaiser et al., 2013). However, in the routine clinical setting there is currently no alternative to iFISH for the detection of copy number abnormalities.

Whole genome DNA analysis using comparative genomic hybridization was a significant technical development in molecular cytogenetics (Kallioniemi et al., 1992). A parallel technology utilizing single nucleotide polymorphism (SNP) arrays has been used to detect copy number changes and loss of heterozygosity (Mullighan et al., 2007). Cost and turnaround time renders array-based assays difficult in a diagnostic setting. Recently, multiplex ligation-dependent probe amplification (MLPA) was developed as a fast and robust alternative method to analyze copy number changes in a wide set of loci. It is based on a multiplex PCR method and is able to detect relative copy number changes affecting small regions (55–80 nucleotides) and single nucleotide aberrations in up to 46 genomic DNA regions per multiplex reaction (Schouten et al., 2002; Alpar et al., 2013). Specific panels have been recently developed for several disease entities such as inherited conditions and hematological malignancies including myeloma (Alpar et al., 2013). To date, MLPA has only been compared to iFISH outside clinical trials (Alpar et al., 2013). In this work, our aim was to evaluate a myeloma-specific commercially available set of MLPA probes against the use of either SNP arrays or iFISH for the detection of copy number abnormalities. The ultimate aim of this work is to determine the performance of an all molecular diagnostic approach, comprising MLPA for copy number abnormality and qRT-PCR for translocation assessment.

**MATERIALS AND METHODS**

**The Myeloma IX Study**

The MRC Myeloma IX trial recruited 1,970 newly diagnosed patients. The trial design and results have been reported previously (Morgan et al., 2012). In brief, the trial included two treatment pathways: the intensive treatment pathway for younger and fitter patients and the nonintensive pathway for older and less fit patients. The intensive pathway comprised high dose melphalan and autologous transplantation after induction with cyclophosphamide, thalidomide, and dexamethasone (CTD) or cyclophosphamide, vincristine, doxorubicin, and dexamethasone. The nonintensive pathway consisted of a randomization to either attenuated CTD (CTDa) or melphalan and prednisolone. All patients were subsequently randomized to thalidomide maintenance or no thalidomide maintenance. The trial was approved by the MRC Leukaemia Data Monitoring and Ethics committee (MREC 02/8/95, ISRCTN68454111).

**Patient Samples**

Bone marrow aspirates from newly diagnosed patients with multiple myeloma, entered into the UK MRC Myeloma IX study, were obtained after informed consent. PCs were selected using CD138 microbeads and magnet-assisted cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany) as described previously and PC purity was confirmed to be >90% in all cases by cytospin. RNA and DNA were extracted using commercially available kits (RNA/DNA mini kit or Allprep kit; QIAGEN) according to manufacturers’ instructions.

**iFISH Analysis**

Interphase FISH analysis was performed on CD138-selected PCs using the micro-iFISH technique and probes that have previously been documented (Boyd et al., 2012). Briefly, probes to detect translocations (t(4;14), t(6;14), t(11;14), t(14;16), t(14;20)), copy number abnormalities...
Array Analysis
GeneChip Mapping 500K Array set (Affymetrix) were performed as previously described. Twenty additional samples in this study had also been analyzed using SNP6. For mapping array data, the SNP inferred copy number were obtained using GTYPE and dChip, as previously described. Data have previously been deposited as GEO accession number GSE21349. Homozygous deletions were identified as having an inferred copy number less than 0.7, hemizygous deletions between or equal to 0.7–1.4, and gains > 2.4 for 500K arrays. Similarly, for SNP6, homozygous deletions were defined as a ratio less than 1.6, hemizygous deletions as a ratio between or equal to 1.6 and 2.4, and gains if the ratio was greater than 2.4.

MLPA Analysis
Fifty nanograms of DNA were subjected to SALSA MLPA P425-B1 multiple myeloma probemix developed by MRC-Holland (Amsterdam, The Netherlands). The probemix contained 46 probes for the following regions (genes): 1p32.3 (CDKN2C-FAF1), 1p31.3–p31.2 (LEPR and RPE65), 1p21.3–p21.1 (OPYD and COL11A), 1p12 (FAM46C), 1q21.3 (CKS1B), 1q23.3 (NUF2 and PBX1), 5q31.3 (PCDHA1, PCDHAC1, PCDHBB, PCDHB10, SCL25A2, and PCDHGA11), 9p24 (JAK2), 9q34 (COL5A1) 12p13.31 (CHD4, VAMP1, CD27, and NCAPD2), 13q14 (RB1, DLEU1, and DIS3), 13q22.1 (DIS3), 14q32.32 (TRAF3), 15q12 (GABRB3), 15q26 (JGFR1), 16q12 (CYLD), 16q23 (WWOX), and 17p13 (TP53). In addition, this probemix contained 11 reference probes, locating in genomic regions that are relatively stable in multiple myeloma, allowing reliable normalization and data analysis of the results. MLPA reactions, including internal quality controls and negative controls, were performed according to the manufacturer’s instructions. The PCR products were analyzed using an ABI 3730 DNA analyser (Life Technologies, Paisley, UK) and Coffalyser.net software (MRC Holland, Amsterdam, The Netherlands). Copy number at each locus was estimated as described previously (Schwab et al., 2010). In summary, values above 1.2, between or equal to 1.2 and 0.75, between or equal 0.75 and 0.25, and below 0.25 were considered as gain, normal, hemizygous loss, and homozygous loss, respectively. Values above 1.6 were consistent with amplification (more than three copies).

Statistical Analysis
Performance of the classification function of iFISH and MLPA were measured against SNP arrays using the R Caret package. Survival curves were plotted using the Kaplan–Meier method. Differences between curves were tested for statistical significance using the log-rank test, with \( P < 0.05 \) taken as the level of significance.

RESULTS
Frequency of Abnormalities Detected by iFISH, SNP Arrays, and MLPA
Eighty six patients were assessable for a comparison between iFISH, MLPA, and 500K arrays. Copy number abnormalities (including del(1p32), gain(1q21), del(13q), del(16q), del(17p), and hyperdiploidy) were identified in 60 patients using 500K arrays (70%), 76 patients (88%) using iFISH, and 67 patients (78%) using MLPA. The frequency of each individual abnormality is given in Table 1. There was good concordance of results between different assays with the iFISH and MLPA giving more consistent results than with mapping arrays, which generally underestimates the frequency of abnormalities. The most discordant results we
obtained were seen with del(17p) (3.5% for mapping array, 7% for iFISH, and 4.6% for MLPA). However, this reflects a difference of one patient given the low frequency of del(17p) in the patient cohort. Adverse prognostic lesions such as gain(1q21), del(1p32), and del(17p) were found in 38% of patients by MLPA, 32% by 500K arrays, and 43% by iFISH. Multiple adverse aberrations (del(1p32), gain(1q21), and del(17p)) were present in several patients, as previously described, resulting in cosegregation in 12, 9, and 14% of patients by MLPA, 500K arrays, and iFISH, respectively.

Comparison of MLPA and SNP Arrays

Eighty six samples were assessable for MLPA, 500K arrays, and iFISH. When MLPA and iFISH were compared to 500K arrays, MLPA was associated with better sensitivity and specificity than iFISH for the different regions analyzed (Table 2). The sensitivity and specificity of MLPA in detecting del(17p) deletions were 100 and 99% versus 100 and 97% for iFISH, respectively. Given the small number of del(17p) in this dataset, an additional comparison was made between MLPA and a series of samples enriched for PC leukemia for which SNP6 data were available (n = 29). Twelve samples had del(17p) in this dataset and the sensitivity of MLPA was 94% and the specificity 100% when compared to SNP6 arrays (Table 3). For CDKN2C, located at 1p32, losses were detected with a sensitivity and specificity of 100 and 97% versus 100 and 93% for iFISH. In the expanded analysis using the SNP6 dataset, the sensitivity of MLPA for CDKN2C was 83% and the specificity 100%. The sensitivity of MLPA to detect WWOX deletions was 100% both for MLPA and iFISH but the specificity of MLPA was greater (92% vs. 84%). The loss of CYLD is not assessed by common iFISH probes but MLPA was able to detect them with good sensitivity (100%) and specificity (93%) when compared to 500K array. The sensitivity of detecting del(13q) by MLPA was lower than iFISH (97% vs. 100%) but MLPA yielded better specificity (77% vs. 71%). To facilitate comparisons, we defined hyperdiploidy in the three methods as being the gain of at least two chromosomes among chromosome 5, 9, and 15. The sensitivity of detecting hyperdiploidy was 100%, but MLPA lacked specificity (80%) like iFISH (75%) suggesting both methods currently overestimate hyperdiploidy. In addition, compared to iFISH, MLPA offers additional information regarding other regions of interest in myeloma (FAF1, CYLD, TRAF3, and FAM46C) with a very good concordance of results compared to SNP arrays (Table 2).

Comparison of MLPA and iFISH

One hundred and seventy-one patients derived from the Myeloma IX study had both iFISH and
MLPA data available. When compared to iFISH, specificities were good (Table 4). The specificity of MLPA to detect TP53 (del(17p)) deletions and FAF1-CDKN2C (1p32) deletions were 99% but the sensitivities were lower (76 and 80%, respectively). Similar results were seen with 1q21 (CKS1B), 13q (RB1), 16q (WWOX), and hyperdiploidy confirming the results observed when compared to the 500K arrays and suggesting MLPA could underestimate the number of lesions.

As MLPA can deliver a semiquantitative assessment, one can determine the relative number of copies of CKS1B and, therefore, differentiate between gain and amplification of 1q21. By applying k-mean clustering to the average of two probes for CKS1B in 264 patients, we were able to identify normal patients with two copies of CKS1B (ratio range from 0.83 to 1.19, center = 1.02), patients with a gain or three copies of CKS1B (range 1.2–1.59, center 1.36), and a small subset of patients with CKS1B amplification (range 1.63–2.53, center 1.86). Panel B and C: Survival analysis. Survival data were available for the 176 Myeloma IX samples and suggests that amplification is associated with a worse outcome in terms of both PFS (Panel B) and OS (Panel C).

**TABLE 4. Sensitivities and Specificities versus iFISH and CGH Arrays: MLPA Compared to iFISH for the Detection of Copy Number Changes (n = 171)**

| Gene (locus) | Sensitivity (%) | Specificity (%) | Frequency by iFISH (%) | Frequency by MLPA (%) |
|-------------|----------------|----------------|------------------------|-----------------------|
| TP53 (17p)  | 76             | 99             | 15                     | 12                    |
| CDKN2C (1p32) | 80             | 99             | 17                     | 15                    |
| CKS1B (1q21) | 79             | 94             | 44                     | 39                    |
| Hyperdiploidy (gain of 5–9-15) | 79             | 94             | 57                     | 40                    |
| RB1 (13q14)  | 91             | 95             | 55                     | 50                    |
| WWOX (16q23) | 72             | 98             | 27                     | 21                    |

Figure 1. MLPA for identification of gains and amplifications of CKS1B was associated with a negative impact on survival in this small dataset. Panel A: Distribution of CKS1B ratios. By applying k-mean clustering to 264 MLPA CKS1B raw values, we were able to identify normal patients with two copies of CKS1B (ratio range from 0.83 to 1.19, center = 1.02), patients with a gain or three copies of CKS1B (range 1.2–1.59, center 1.36), and a small subset of patients with CKS1B amplification (range 1.63–2.53, center 1.86). Panel B and C: Survival analysis. Survival data were available for the 176 Myeloma IX samples and suggests that amplification is associated with a worse outcome in terms of both PFS (Panel B) and OS (Panel C).
identify patients with two copies of CKS1B (ratio range from 0.83 to 1.19, center = 1.02), patients with a gain (three copies) of CKS1B (range 1.2–1.59, center 1.36), and a small subset of patients with CKS1B amplification (range 1.63–2.53, center 1.86). The specificity in detecting amplification using a ratio >1.6, when compared to patients with more than three copies of CKS1B by iFISH, was good (96%). The sensitivity was nevertheless low (56%). Of note, all the false positives were in fact gained but one bore a subclonal amplification by iFISH (39% of cells). We were also able to show that amplification of 1q21 was present in 8.5% of patients at diagnosis and associated with a negative impact on survival in this small dataset, Figure 1.

### Results of an All Molecular Approach for the Detection of High Risk Myeloma

We went on to implement this diagnostic strategy in combination with our previously described expression-based assay for the detection of recurrent translocations involving the IGH locus (Kaiser et al., 2013). In a series of 154 patients from the Myeloma IX trial, which were analyzed by qRT-PCR for translocations and MLPA for copy number abnormality, 53% of patients had at least one adverse lesion defined as the presence of a t(4;14), t(14;16), t(14;20), del(1p32), gain(1q21), and del(17p). Patients with an adverse prognostic lesion did significantly worse than those with none with a median Progression-Free Survival (PFS) of 14.9 (95% CI 13.2–19.9) versus 23.0 (95% CI 18.5–30.1) months and a median Overall Survival (OS) of 35.8 (95% CI 38.3–62.1) versus 47.6 (95% CI 28.8–43.7) months (Figure 2). These data suggest that an all molecular approach can be used to define outcome.

### DISCUSSION

Over the last 15 years, it has become clear that specific genetic lesions have an importance on the tumor biology of myeloma. Increasing evidence suggests that myeloma is not a single disease but a collection of diseases with distinct clinical behaviors. Understanding this heterogeneity will enable us to perform precision medicine where clinical decisions are based on molecular subtypes of disease. Identifying these groups and designing clinical trials for them will refine our current management strategies, avoiding overtreating or undertreating specific subgroups (ClinicalTrials.gov, n.d.). The first step toward precision medicine is applying a risk stratification approach based on prognostically important lesions. Translocations were first identified as being prognostic with more recent studies emphasizing the role of copy number changes such as gain(1q21), del(1p32), and del(17p) (Avet-Loiseau et al., 2011; Boyd et al., 2012; Avet-Loiseau et al., 2012, 2013; Hebraud et al., 2014). To increase the sensitivity and specificity of such approaches for the demonstration of clinical outcomes (Boyd et al., 2012), it is important to detect all the prognostically relevant lesions present in a myeloma cell, an aim which is technically difficult with iFISH.

Although RNA-based classifications have been developed to identify translocations (Bergsagel and Kuehl, 2005; Kaiser et al., 2013), there is currently no equivalent molecular approach to detect...
copy number changes in a routine clinical setting. MLPA is a cost effective and robust method that can analyze up to 50 independent genetic loci in a single reaction. MLPA has been used in multiple diagnostic settings in both benign and malignant conditions, such as neurogenetic disorders, lymphoma, and acute leukemia. (Coll-Mulet et al., 2008; Schwab et al., 2010; Donahue et al., 2011; Alpar et al., 2013). We show that MLPA is applicable to sorted CD138 myeloma cells where it is a highly effective tool to accurately access a wide panel of copy number abnormalities present at diagnosis when compared to SNP arrays. Discrepancy between iFISH and MLPA probably relate to point mutations, cryptic lesions, or subclones, as previously shown in chronic lymphocytic leukemia (Véronèse et al., 2013). The comparison of MLPA to mapping arrays demonstrates the power of this method, as for each lesion the sensitivity and specificity is better than when using iFISH.

Importantly, MLPA can be used to determine the number of copies of each region, which is of clinical relevance as amplification of **CKS1B** is associated with disease progression (Sawyer et al., 2014). We show for the first time that amplification of **CKS1B** is present in approximately 8% of patients at diagnosis and has a negative impact on survival. No high amplification samples were seen in our dataset (maximum five inferred copies). As far as the prognostic impact of the other lesions is concerned the exact prognostic relevance of each individual lesion remains to be determined, but our analysis suggests that MLPA may be used in combination with expression assays to determine a molecular risk stratification in myeloma, thus providing a rapid and robust alternative to iFISH.

MLPA also offers additional information in comparison to iFISH. By tilting other regions of interest, MLPA broadens the spectrum of analysis. MLPA covers 15 regions: some covered by standard FISH panels (**TP53**, **CDKN2C**, etc.) and some other ones that bear a more mechanistic relevance such as **CYLD** and **TRAF3**. The latter are associated with NF-κB activation, a feature that correlates with an aggressive disease phenotype and may constitute an interesting new target in myeloma (Annunziata et al., 2007).

As far as cost is concerned, although they are likely to vary between centers and the number of samples processed, the estimated cost of this MLPA panel is roughly 60–70 € which is much less than the estimated cost of iFISH (six probes 750 €) and SNP arrays (350–500 €).

Thus, MLPA offers a powerful alternative to iFISH which in its current format is able to determine copy number abnormalities at 15 independent loci. We show that MLPA can accurately determine recurrent copy number abnormalities and that it can be readily applied in a diagnostic laboratory at low cost.

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