European Journal of Cancer (2013) xxx, xxx–xxx

Available at www.sciencedirect.com

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journal homepage: www.ejcancer.com

Rapid identification of compound mutations in patients with Philadelphia-positive leukaemias by long-range next generation sequencing

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KEYWORDS
NGS
CML
ALL
BCR-ABL1
Tyrosine kinase domain
TKI

Abstract An emerging problem in patients with Philadelphia (Ph)-positive leukaemias is the occurrence of cells with multiple mutations in the *BCR-ABL1* tyrosine kinase domain (TKD) associated with high resistance to different tyrosine kinase inhibitors. Rapid and sensitive detection of leukaemic subclones carrying such changes, referred to as compound mutations, is therefore of increasing clinical relevance. However, current diagnostic methods including next generation sequencing (NGS) of short fragments do not optimally meet these requirements. We have therefore established a long-range (LR) NGS approach permitting massively parallel sequencing of the entire TKD length of 933 bp in a single read using 454 sequencing with the GS FLX+ instrument (454 Life Sciences). By testing a series of individual and consecutive specimens derived from six patients with chronic myeloid leukaemia, we demonstrate that long-range NGS analysis permits sensitive identification of mutations and their assignment to the same or to separate subclones. This approach also facilitates readily interpretable documentation of insertions and deletions in the entire BCR-ABL1 TKD. The long-range NGS findings were reevaluated by an independent technical approach in select cases. Polymerase chain reaction (PCR) amplicons of the BCR-ABL1 TKD derived from individual specimens were subcloned into pGEM®-T plasmids, and >100 individual clones were subjected to analysis by Sanger sequencing. The NGS results were confirmed, thus documenting

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http://dx.doi.org/10.1016/j.ejca.2013.11.030

Please cite this article in press as: Kastner R. et al., Rapid identification of compound mutations in patients with Philadelphia-positive leukaemias by long-range next generation sequencing, *Eur J Cancer* (2013), http://dx.doi.org/10.1016/j.ejca.2013.11.030

the reliability of the new technology. Long-range NGS analysis therefore provides an economic approach to the identification of compound mutations and other genetic alterations in the entire BCR-ABL1 TKD, and represents an important advancement of the diagnostic armamentarium for rapid assessment of impending resistant disease.

1. Introduction

Mutations in the BCR-ABL1 tyrosine kinase domain (TKD) are regarded as the most important mechanism of resistance to tyrosine kinase inhibitors (TKIs) in patients with Philadelphia (Ph)-positive leukaemias [1–4]. A variety of methods are currently used for the detection of mutations in the BCR-ABL1 TKD ranging from broad screening techniques to highly sensitive detection methods for specific mutations [5–13]. The heterogeneity of diagnostic approaches accounts, at least in part, for the differences in reported frequencies of mutations [3]. Mutational screening is most commonly performed by bidirectional Sanger sequencing of the entire BCR-ABL1 TKD amplified by polymerase chain reaction (PCR), which does not reveal the presence of mutant subclones representing less than 10–20% of the Ph-positive cell pool [14]. Despite this limitation, Sanger sequencing is currently recommended as the method of choice by the European Leukaemia Net (ELN) [14–17]. A number of alternative methodologies providing higher sensitivity have been introduced to facilitate earlier detection of mutant, potentially resistant BCR-ABL1 cells, and some of these approaches permit accurate monitoring of the size of mutant subclones during therapy [6,9,11,13,18–21]. Our observations based on a quantitative ligation-dependent (LD)-PCR technique revealed that mutant subclones may appear and expand rapidly after onset or change of TKI-therapy, and subclone-specific response to treatment can be readily documented [19]. Surveillance of the size of mutant subclones during TKI-treatment was shown to provide information on their responsiveness to therapy and the presence or imminent onset of resistant disease [19–21]. Although various methods for mutational analysis based on PCR-mediated pre-amplification of the BCR-ABL1 TKD permit highly sensitive subclone detection, sequence errors introduced by the reverse transcriptase and PCR polymerase must be considered (data not shown). Owing to this phenomenon, the practical detection limit of prospective screening for mutant subclones after PCR amplification of the BCR-ABL1 TKD is in the range of 1%. Although several techniques can reveal the presence of mutations even in considerably smaller cell subsets, reliable distinction between true mutations and errors introduced by the indicated enzymes is not always feasible. This restriction also applies to massively parallel sequencing using next generation sequencing (NGS), as indicated by bioinformatic analysis [22].

Recent observations suggest that occurrence of the so-called compound mutations, defined by the presence of two or more mutations on the same DNA molecule, is often associated with particularly high resistance to multiple TKIs [20,23]. The frequency of compound mutations is apparently quite high, thus rendering their reliable detection an important diagnostic challenge [20,23]. Sensitive and quantitative methods such as LD-PCR only have the capacity to identify compound mutations by the documentation of concordant kinetics of different mutant subclones in certain instances. NGS has therefore become the emerging method of choice for sensitive sequencing of the BCR-ABL1 TKD. Due to the limited read-length offered by most current NGS technologies, multiple overlapping amplicons are required to cover the entire TKD. This prevents the assignment of nucleotide substitutions located on different amplicons to the same DNA molecule, and therefore requires additional laborious steps to facilitate unequivocal identification of constellations with compound mutations [20]. We have therefore addressed the possibility to overcome this disadvantage by using the GS FLX+ platform (454 Life Sciences) with an average read-length of 900 bp to cover the complete BCR-ABL1 TKD in a single read.

2. Materials and methods

2.1. Isolation of RNA, reverse transcription and PCR amplification of the BCR-ABL1 TKD for long-range (LR)-NGS and fragment subcloning

Primary nucleic acid analyte material was extracted from peripheral blood samples following standard total RNA extraction methods (QIAamp RNA Blood Mini Kit, Qiagen, Hilden, Germany or Eurobio, Les Ulis, France), and cDNA was synthesised using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies, Carlsbad, United States of America (USA)) according to the manufacturers' recommendations.

For amplification of the BCR-ABL1 kinase domain, a two-step semi-nested RT-PCR was performed, as described previously [7]. The Expand Long template PCR system (Roche, Basel, Switzerland) was used for amplification of PCR products for subsequent LR-NGS analysis. For the subcloning/sequencing strategy performed, an additional alternative proof-reading enzyme, the Phusion High-Fidelity DNA Polymerase (New England Biolabs, Massachusetts, USA) was applied.

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