Oligonucleotide-based Array CGH as a Diagnostic Tool in Multiple Myeloma Patients

Využití techniky komparativní genomové hybridizace na oligonukleotidových čipech jako diagnostického nástroje u pacientů s mnohočetným myelomem

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Summary

Multiple myeloma (MM) is a hematological disease caused by malignant proliferation of clonal plasma cells (PCs) known for its clinical and biological heterogeneity. Identification of chromosomal changes in genome of PCs plays a key role in MM pathogenesis and is supposed to have important prognostic significance for MM patients. There are two major genetic entities in MM. Hyperdiploid tumors (H-MM), which include about 50% of MM tumors, often have multiple trisomies involving chromosomes 3, 5, 7, 9, 11, 15, 19, and 21 and a substantially lower prevalence of *IgH* translocations. Nearly half of tumors are non-hyperdiploid (NH-MM), and mostly have one of five recurrent *IgH* translocations: 11q13 *(CCND1)*, 6p21 *(CCND3)*, 16q23 *(MAF)*, 20q12 *(MAFB)*, and 4p16 (*FGFR3* and *MMSET*). The development and expanded use of new technologies, such as genome-wide array-based comparative genomic hybridization (aCGH) has accelerated genomic research in MM. This technique is a powerful tool to globally analyze recurrent copy number changes in tumor genome in a single reaction and to study cancer biology and clinical behaviors. It widely overcame routinely used cytogenetic techniques (G-banding, FISH) both in minimal resolution of chromosomal changes and amount of obtained genomic data important for further analyses and clinical applications. Array CGH technique is now used to better understanding of molecular phenotypes, sensitivity to particular chemotherapeutic agents, and prognosis of these diseases. This paper brings brief literature and methodic overview of oligonucleotide-based array-CGH technique in MM diagnosis.

Key words

multiple myeloma – array-CGH – cytogenetics

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Souhrn

Mnohočetný myelom (MM) je hematologické onemocnění způsobené maligní proliferací klonálních plazmatických buněk (PCs), které se vyznačuje značnou klinickou a biologickou variabilitou. Identifikace chromozomových změn v genomu PCs hraje klíčovou roli v patogenezi MM a má také důležitý prognostický význam u pacientů s MM. Z genetického hlediska lze MM rozdělit na dva subtypy. Hyperdiploidní MM (H-MM), který se vyskytuje u 50 % případů, je charakterizován častou incidencí trizomií chromozomů 3, 5, 7, 9, 11, 15, 19 a 21 a dále nízkým výskytem translokací IgH. Téměř polovina případů je klasifikována jako non-hyperdiploidní MM (NH-MM), u kterého lze často najít jednu z pěti rekurentních translokací IgH: 4p16 (*FGFR3* a *MMSET*), 6p21 (*CCND3*), 11q13 (*CCND1*), 16q23 (*MAF*), 20q12 (*MAFB*) a který je asociován s nepříznivou prognózou onemocnění. Rozvoj a rozšířené využívání nových technologií, jako je technika celogenomové komparativní genomové hybridizace na oligonukleotidových čipech (aCGH), výrazně posunula výzkum genomových změn u MM, jelikož umožňuje v rámci jedné reakci analýzou chromozomových změn v celém genomu, a tak představuje ideální nástroj pro studium nádorové genetiky a je vhodnou aplikací pro rutinní analýzy v klinické praxi. Technika aCGH významně překonává běžně používané cytogenetické techniky (G-pruhování, FISH), a to jak v možnostech minimálního rozlišení chromozomových změn, tak i v kvalitě a množství získaných genomických dat nezbytných pro další analýzy a klinické aplikace. Technika aCGH je nyní používána k lepšímu pochopení molekulárního fenotypu nádorových buněk, pro studium vlivu chromozomových změn na citlivost na určitá chemoterapeutika a prognózu onemocnění. Tento dokument přináší stručný metodický a literární přehled použití techniky oligonukleotidové aCGH v diagnostice MM.

Klíčová slova

mnohočetný myelom – array-CGH – cytogenetika

Introduction

Incidence of specific chromosomal aberrations (CHA) in genome of malignant plasma cells (PCs) is considered to be one of the most important independent prognostic factors for patients with MM [1]. These changes are often observed in chromosomal areas where tumor suppressor genes or oncogenes are located [2]. It is well established that loss of tumor suppressor genes or copy gains of oncogenes are essential events in developmental process of malignant PCs [3,4]. Several studies show that incidence of specific CHA is an important prognostic factor often connected with response to chemotherapy or bone marrow transplantation [5,6]. Thus, detection of CHA is an essential step in determination of a given MM patient pathogenesis. Effective detection of these changes by means of standard karyotyping using G-banding cytogenetic techniques is limited (about 30% of cases) due to low resolution (5–10 Mb) and low proliferation of PCs. This limitation can be overcome by newer techniques, such as fluorescent *in situ* hybridization (FISH). However, this technique detects only a limited number of target sequences in the genome. Using FISH, overall detection rate of CHA reaches over 90% of all cases [7,8]. In MM patients, routine application of FISH in clinical practice is mainly focused on identifying specific chromosomal aberrations. Several studies described prognostic significance of del(13)(q14)/loss of chromosome 13, del(17)(p13), gain(1)(q21) and *IgH* translocations [7,8–10], but current understanding of MM pathogenesis together with development of modern genome-wide screening techniques proves that FISH is insufficient for description of MM genetic heterogeneity. Introduction of microarray-based comparative genomic hybridization (aCGH) technique by Solinas-Toldo et al [11] gives researches an ideal molecular cytogenetic tool, allowing detection of CHA including deletions and duplications in a single experiment.

Oligonucleotide-based aCGH is a robust modern technique, which allows detecting of CHA of size as low as 2kb in a single reaction during 40 hours depending on array platform (Tab. 1). Such resolution gives us a possibility for detection of CHA on exon level [12,13], which dramatically improves our investigations of chromosomal rearrangements in genome of malignant cells. Moreover, with help of modern, sophisticated sta-

Tab. 1. Comparison of different cytogenetic and aCGH techniques according to resolution of the technique. Adapted from [13] and Agilent Technologies, Inc.

		Resolution	Coverage
a) Cytogenetics	Karyotyping	> 10 Mb	Complete
	SKY	> 2 Mb	Complete
	Traditional CGH	> 2 Mb (cytoband)	Complete
	FISH (interphase)	≥ 20 Kb	Probe Specific
	FISH (metaphase)	≥ 100 Kb	Probe Specific
b) aCGH	BAC	100 Kb (Spectral Genomics – 2 Mb)	Complete
	cDNA	2 Kb	Genes Only
	Oligo (60-mer)	0,06 Kb	Complete

tistic tools for CHA evaluation, it is possible to interpret data mined from aCGH technique quite fast and easy, correlate these data with other techniques or with clinical features. Thus, aCGH technique and genomic copy number analyses with SNP genotyping arrays are proving particularly effective for investigations of important CHA connected with cancer diseases in molecular level [14].

Methodology of Oligonucleotide-based aCGH Technique

In many hematological malignancies, aCGH technique has been often successfully used for detection of genomic alterations [15–17]. An essential issue in the implementation and optimization of aCGH technique in our laboratory was obtaining sufficient amount of genomic DNA (gDNA) from patients' PCs samples. For successful aCGH experiment and confident interpretation of aCGH results in MM diagnosis, it is necessary to get purified gDNA from population of a malignant clone of PCs, which is obtained by immunomagnetic (MACS) or immunofluorescent (FACS) separation from patient's bone marrow. Malignant PCs are identified by surface antigenic markers of CD 138+ CD45–[18,19]. For further differentiation of abnormal from healthy PCs populations, CD38+, CD19+, CD56+ markers are used [20]. After sorting, samples of high purity with at least $0{,}5 \times 10^6$ PCs from abnormal population are obtained. For aCGH, minimum infiltration of malignant PCs in the sample should be above 90%. Minimum content of tumor cells represented in the sample for aCGH, which is capable of distinguishing malignant from normal cells, is around 20–30% [21]. Then, isolation of gDNA can be made by typical phenol-chloroform extraction or using a variety of commercially available kits. In our laboratory, we commonly use Gentra Puregene Core Kit A (Qiagen). Regardless of isolation method, it is necessary to obtain high-quality gDNA as input. It minimizes the risk of abnormal array background and generally is the cornerstone for quality of whole genome analysis. As standard procedure, gDNA should be checked using gel electrophoresis and measured for yield and purity by a spectrophotometer. Recommendations according to Agilent protocol suggest the ratio of absorbance 260 nm/280 nm in the range of 1.75 to 1.90 and 260/230 greater than 2.0. As standard, minimum amount of gDNA for aCGH experiment is 0.5 µg. In our laboratory, we use 4x44K format 1 µg of gDNA. Concentration of input gDNA should be between 200–400 ng/µl.

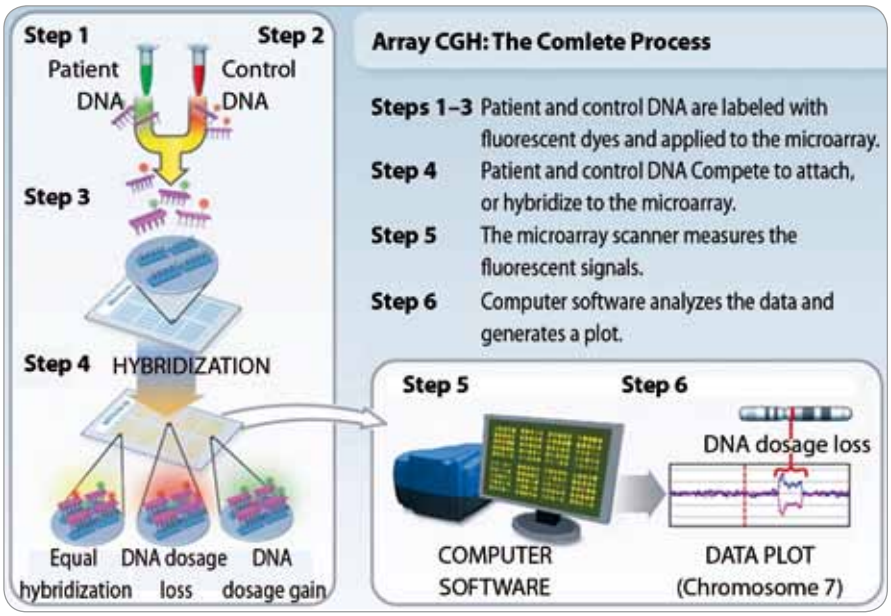

Fig. 1. Scheme of typical genome-wide aCGH experiment. Adapted from [23].

In our laboratory, we use genome-wide oligonucleotide-based aCGH 4x44K (Human HD-CGH 4x44K, Agilent Technologies) platform together with complete hardware and software equipment required for aCGH technique from Agilent (Agilent Technologies, Santa Clara, USA). The principle of aCGH is hybridization between the "probe" and DNA fragments (60 bp DNA fragments defined) "spotted" using photolithography on a glass matrix (= array) [22]. Probe DNA is prepared from the DNA sample and healthy control DNA. DNA fragments of known sequence are located on exact spots on the array, and each probe is associated with specific position in the genome. Genomic DNA is obtained from various sources of material (cultured cells, tissues, blood) cut by restriction enzymes and labeled with fluorescent dyes of different color, typically red and green. Samples and DNA controls are cohybridised, incorrectly linked probes removed during wash step and complementary probes that remained attached to the array provide the fluorescent signal, which is acquired by a sensitive scanner. Acquired digital "picture" from scanner is the analyzed by appropriate software. Flow-chart of typical aCGH experiment is shown on Fig. 1 [23].

Routine aCGH experiment begins with digestion step, where gDNA is digested by restriction enzymes (Alu1, Rsa1, DNaseI) into fragment size of 200–2,000 bp. For fluorescent labeling of sample and control DNA, several commercially available kits can be used. Specific activities (= measuring of labeling effectiveness of DNA fragments) will slightly vary according to the manufacturer and type of fluorescent dyes. We use Bioprime Total Genomic Labeling System (Invitrogen) with specific fluorescent dyes Alexa3 and Alexa5. According to manufacturer's protocol, specific activity of labeling with 1 µg gDNA as input should be in the range of 90–100 pmol/µg for Alexa3 and 70–90 pmol/µg for Alexa5. Total DNA yield is expected between 4–7 µg. Labeled samples and controls of the same sex and similar specific activity are mixed together, reaction and hybridization buffer together with unmarked human Cot-1 DNA are then added into reaction and after denatu-

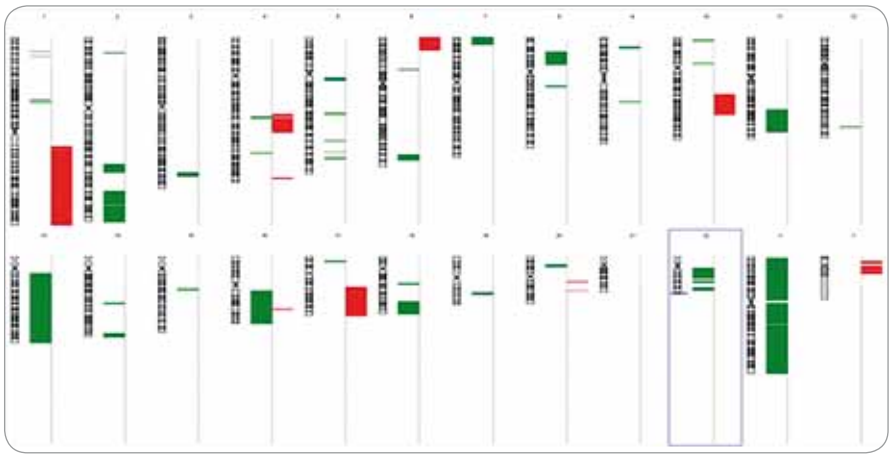

Fig. 2. Example of genome-wide screening of NH-MM patient using aCGH technique. Green color corresponds with areas of loss of genetic material, areas marked with red color are considered as gains of genetic material.

ration and incubation at 95 C/3 min and 30 min/37 C respectively, mixed solution is applied onto the array. Hybridization process of 4x44K array lasts approximately 24 hours at 65 C. After hybridization, array undergoes washing procedures (Wash buffer 1 and 2, Agilent Technologies), and then it is ready for scanning in appropriate scanner. The output of the scanner is a data file, which is further processed by software (Agilent Feature Extraction 9.5.3), which visualizes the data for checking of overall array quality and converts raw data from the scanner into a format suitable for further software applications designed to detect and analyzing genomic changes, such as Agilent Genomic Workbench. The rate of gain or loss of genetic material is obtained by analysing the ratio of green and red fluorescence from the probes (fixed base modal ratio) and selected on the basis of statistical methods (Z-score, ADM, HMM, CBS). Genome without CHA is characterized by yellow fluorescence (log2 = 0). Areas with gained genetic material are characterized by log2 > 0, whereas the values log2 < 0 are considered as loss of genetic material. Graphical image of analysis of CHA using aCGH software are shown in Fig. 2.

Utilization of aCGH Technique in Multiple Myeloma

The introduction of new molecular cytogenetic techniques (FISH, aCGH) brought new possibilities for detection of CHA in MM. Using FISH and especially designed probes, the most frequently CHA were observed in 90% of patients [4,5–8]. According to the International Myeloma Working group (IMWG), current minimal clinical panel of CHA proposed for testing by FISH in MM patients includes 3 aberrations ((del(17)(p13), t(4;14)(p16;q32), t(14;16)(q32;q23)) with known negative effect on MM prognosis. Expanded panel of FISH markers includes hyperdiploidy (trisomies of chromosome 5, 9 and 15), del(13)(q14)/monosomy 13, gain(1)(q21) and t(11;14)(q13;q32) [24]. However, with given heterogeneity of MM, it seems insufficient. Moreover, several studies proved 100% occurrence of chromosomal aberrations in MM when aCGH technique was used [21,25] and therefore utilization of genome-wide techniques in clinical practice is already taken under consideration in IMWG recommendations for cytogenetic analyses used in MM diagnostics.

New technologies, especially oligonucleotide-based comparative genome hybridization, have dramatically changed human genomic analysis by combining the targeted high-resolution aspects of FISH and the genome-wide scale of karyotyping. The first whole genome studies in MM performed by conventional CGH revealed new changes undetectable by G-banding and pointed out aberrations studied by FISH. The most frequent aberrations were: gain 1q, 3q, 9q, 11q, 15q, loss of 6q21, 13q, 14q, 16q [26,27]. The German group [28] for the first time evaluated sensitivity (80.7%) and specificity (97.7%) between CGH and FISH in MM diagnosis. Gutiéréz et al [29] found a significantly shorter overall survival for MM patients with deletions in the genome compared to patients with gain of genetic material. The first use of array-based CGH in MM was published by Largo et al [23], who have verified previous studies made by conventional CGH, clarified the definition of both hyperdiploid and non-hyperdiploid MM subtypes according to FISH and further defined two genetically different subclasses of the H-MM according to the incidence of 7p gain. Using aCGH and GEP (gene expression profiling) analysis and subsequent integration of the results from both techniques, Carrasco et al [30] described two subclasses of H-MM with different prognosis among cohort of 67 newly diagnosed patients with MM. In patients with a combination of hyperdiploidy and trisomy of chromosome 11, a trend for better prognosis was found in contrast to patients without trisomy 11. Moreover, within H-MM subgroup without +11, statistically significant difference for the incidence of 1q gain and -13 ($p < 0.001$, $p = 0.019$, respectively) compared to the subgroup with +11 was found. These results confirmed previously published data about importance of 1q gain as an independent prognostic factor in MM [10]. Deletions of several genes involved in NF-kB pathway were described by Keats et al [21]. Genome-wide analysis of 155 patients brought interesting findings that highlight the importance of NF-kB pathway in the pathogenesis of MM. The paper presents findings of 14q32 deletion, which included potential target genes *TRAF3, AMN* and *CDC42BPB*. The authors describe two other less frequent areas with biallelic deletions: 16q12 with genes *CARD15, CYLD*, and 11q22 with genes *BIRC2/cIAP1, BIRC3/cIAP2*. It was interesting that 5 of 13 (38.5%) identified homozygous deletion of genes (*TRAF3, cIAP1, cIAP2*, and *CYLD*) that are involved in negative regulation of NF-kB. To confirm homozygous deletions, they performed FISH investigations and GEP analyses.

The paper further develops the impact of absence of *TRAF3* gene product in overall prognosis and response to drugs used in MM treatment. Authors showed that deletion of *TRAF3* is associated with poor response to dexamethasone, but rather a good therapeutic response to proteasome inhibitors. Furthermore, the authors proposed that *TRAF3* is a tumor suppressor gene, which was verified by reintroduction of functional copies of genes in adenovirus vector into myeloma cell lines. Cell lines with increased activity NF-kB showed a subsequent reduction in growth and increased apoptosis. In contrast, in cell lines with inactive NF-kB, the introduction of the common *TRAF3* showed no change. Study by Chng et al [31] of a group of 131 patients focused on identifying new prognostic regions using aCGH and GEP arrays. Combined GEP and aCGH analysis identified areas with possible prognostic impact. Patients with deletions at 1p31–1p32 or 20p12.3–12.1 were found to have significantly shorter overall survival compared to patients without these deletions (24.5 months vs. 40 months p = 0.01; 20.6 months vs. 40 months p = 0.06, respectively). Survival after relapse was also significantly reduced, but due to a small number of patients, results were not quite statistically significant. Deletion 20p12.3–12.1 was also significantly associated with deletion 17p13 and also showed a strong trend to associate with t(11;14) detected by FISH.

Our preliminary data from a cohort of 84 MM patients performed on Agilent 4x44K arrays so far confirmed previously published data. We found several new genetic events in MM patients and abnormalities of candidate genes. Data from our study will be published elsewhere.

Conclusion

MM remains incurable even today. Introduction of new types of drugs (bortezomib, lenalidomide, thalidomide) and therapeutic strategies significantly prolonged survival of patients with MM and their quality of life during treatment [32]. Similarly to many other types of hematologic malignancies, MM is characterized by numerous structural and numerical CHA involving many oncogenes, tumor suppressor genes or genes involved in signaling pathways important for cell cycle, apoptosis etc. Detection of CHA is one of the most important independent prognostic markers in MM pathogenesis and prognosis for patients [33]. Oligonucleotide-based aCGH technique provides both qualitative and quantitative information about CHA in the genome of malignant PCs in a single experiment. In MM pathogenesis, it is valuable diagnostic and scientific instrument suitable for description of genetic heterogeneity typical for MM providing new opportunities for identification of new biomarkers capable of discerning prognosis.

During the last decade, several important genetic events in MM pathogenesis using conventional CGH or aCGH were described and verified [21,24,29–31]. Presented data emphasize the strengths of high-resolution aCGH results in the detection of genomic alterations associated with development of disease and prognosis of MM patients. However, it is now clear that there is a need for correlation of aCGH findings with other advanced techniques (GEP, methylation analysis). Moreover, newly developed techniques have relegated the importance of aCGH into the background. Recent publications show the new trend in development, leading to sequencing of the second and third generation [34,35], which provides information in a much larger scale than aCGH platform, which is currently available and thus allow us more precise insight into genetic changes in pathology of all malignant diseases.

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