Array Comparative Genomic Hybridization as a Diagnostic Tool in Cancer

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Abstract

The knowledge of the primary origin of tumor is essential in designing an efficient cancer treatment algorithm. Useful diagnostic tools enable determination of primary origin of the tumor; however the majority of them require tissue examination. Recent years, exploration of circulating tumor cells enabled scientists to study different parameters using the painless liquid biopsy. The present study aimed to identify whether aCGH might be used as a diagnostic tool in cancer detecting the primary origin of the tumor. Blood was extracted from healthy individuals and cancer samples and CTCs isolated. DNA extracted from the above samples and aCGH experiments followed. The samples were blinded analyzed and then unmasked to calculate specificity and sensitivity of the method. The sensitivity was 94%, the specificity 88%, while the positive prediction rate of the primary tumor was 72%. aCGH is a powerful tool in cancer diagnosis and treatment plan with high sensitivity and specificity rates. It can be performed from blood sample, which makes it an appropriate method for every patient, mainly for patients with unknown origin of the primary tumor.

Keywords

Cancer of Unknown Primary Origin, Array Comparative Genomic Hybridization, Cytogenetic, Cancer

1. Introduction

Cancer of unknown origin (CUP) referred to metastatic in which the primary tumor has not been identified. The primary tumor may not be detected or it may disappear after having created the metastasis [1]. CUP accounts for approximately 3% - 5% of all malignancies and the median age of diagnosis is 60 years old. The majority of CUP patients (80%) have unfavourable prognosis [2]. Methods to detect primary origin include liquid microscopy evaluation, immuno-
histochemical assays, detection of specific tumor markers and cytogenetics [3].

Chromosomal abnormalities have implications in tumorigenesis since 1960, when the Philadelphia chromosome was linked to chronic myeloid leukemia [4]. The mechanism of triggering cancer is the fused bcr-abl gene, which leads to rapid division of cells [5]. The above rearrangement does not only create a hybrid gene, but also dysregulate other genes. The abnormal expression of genes might contribute to proliferation or inability of repairing mutations [6]. Not only rearrangements but also deletions and duplications are important in cancer. Several losses in tumor suppressor genes or gains of proto-oncogenes contribute to tumorigenesis. Several cancer types are associated with such abnormalities, like Wilm’s tumor [7] or melanoma [8]. Therefore, whole genome cytogenetic profile could be useful in cancer diagnosis.

Array comparative genomic hybridization (aCGH) is a specific molecular cytogenetic method that combines CGH and DNA microarrays and enables whole molecular cytogenetic profiling. It is proved to help identify primary tumors, thus contributing to more efficient therapy protocols [9]. In the present study based on liquid biopsy and particularly on Circulating Tumor Cells (CTCs), aCGH technique was used to identify the origin of the tumor based on a blinded genomic DNA analysis. The technique is not only able to discriminate healthy from cancer samples but also to identify the origin of the tumor.

2. Materials and Methods

2.1. Samples

40 ml of blood was collected from 34 patients suffering from different types of cancer, while the same amount was collected from 9 healthy donors. Blood was placed in sterile 50 ml Falcon tubes (4440100, Orange Scientific, Braine-l’Alleud, Belgium) containing 7 ml of 0.02 M EDTA (E0511.0250, Duchefa Biochemie B.V., Haarlem, The Netherlands). Healthy individuals contained five male and four female samples while the patients’ samples included 16 males and 18 females. Distribution of cancer type in patients group was as follows: breast (8), prostate (4), lung (6), colorectal (4), gastrointestinal (5), ovarian (4) and other cancers including haematological, hepatocellular, melanoma, pancreatic, esophageal, and urothelial. There were no data concerning the stage of cancer. The majority of samples were received from USA (28) and Philippines (5) while there were sent also from Malaysia (2), Germany (1), United Kingdom (1), Canada (1), Poland (2), Israel (2) and South Africa (1). Samples’ age was 61.48 ± 16.04 years old. The samples that were used were collected randomly among cancer and healthy samples. The study was accomplished during January 2018 to May 2019.

2.2. Blood Sample Preparation

Whole-blood samples were centrifuged for 20 min at 2500 × g at RT with 4 ml polysucrose solution (Biocoll separating solution 1077, Biochrom, Berlin, Germany). Mononuclear cells, lymphocytes, platelets and granulocytes were col-
lected after centrifugation and washed with phosphate-buffered saline (PBS) (P3813, Sigma-Aldrich). The cells were incubated in lysis buffer (154 mM NH₄Cl (31107, Sigma-Aldrich), 10 mM KHCO₃ (4854, Merck, Darmstadt, Germany), and 0.1 mM EDTA in deionized water) for 10 min to lyse the erythrocytes. Samples were then centrifuged as above and washed with PBS. Cells from the healthy donor were incubated at 4˚C for 30 min with CD45 magnetic beads (39-CD45-250, Gentaur, Kampenhout, Belgium), whereas those from patients with cancer were incubated with pan-cytokeratin beads (recognizing CK4, CK5, CK6, CK8, CK10, CK13 and CK18) (5c-81714, Gentaur) at 4˚C for 30 min. Following incubation, the samples were placed in a magnetic field to collect microbead-bound cells for pan-cytokeratin and negative selection was performed for CD45 cells, which were washed with PBS. Molecular analysis was performed on the isolated CD45-negative cells (non-cancerous) and the pan-cytokeratin-positive cells (cancerous).

2.3. Array CGH

Genomic DNA was isolated with QIAamp DNA Mini Kit (51306, Qiagen, Hilden, Germany) from the above cells and then, aCGH protocol with Sureprint G3 human CGH 8 × 60 K platform (G4450A, Agilent, CA, USA) followed according to manufacturer’s instruction. The analysis was performed with Cytogenomics. For each abnormality, the genes that were involved on the appropriate locus were further literately studied to identify potential involvement in any type of cancer. Following gene study, the researcher suggested the type of cancer based only on experimental data. Finally, the diagnosis obtained from experimental data was unmasked and compared with that of physicians. In all reactions there were used reference male and female samples as control.

2.4. Statistical Analysis

The data categorized first in two groups, as cancer and healthy and the positive and negative predictive values (PPV-NPV respectively), sensitivity as well specificity were calculated. A second analysis included only cancer samples and PPV was calculated based on specific type of cancer between blinded experimental data and medical form’s data.

2.5. Ethics Approval

This study was not a clinical trial and did not include any interventions in the patients. All procedures were conducted according to the standards of Safety, Bioethics and Validation. The study was reviewed and approved by the Bioethical Committee of the Research Genetic Cancer Centre Group. All patients/donors provided written consent for the use of their samples in the present study. The patients retained the right to withdraw their samples until the date when the sample was received at the laboratory and tested.

3. Results

The samples were firstly classified as cancer and healthy. Cancer samples were
thirty-four while healthy were nine. The aCGH results categorized samples based only on raw data and thirty-two cancer samples predicted as cancerous, while only one normal predicted as cancer. On the contrary, eight healthy samples predicted as normal and two cancer samples predicted as normal. Therefore sensitivity and specificity were calculated based on the above data. The analysis of aCGH data revealed sensitivity 94.11% and specificity 88.88% between healthy and cancer samples. Data are summarized in Table 1. Followed initially classification, cancer samples were further categorized according to their type. The performer predicted the type of cancer based once again on aCGH raw data, and then samples unmasked and the real type of cancer was compared. Among thirty-four cancer samples twenty-five were categorized correctly while in nine samples the type was not correctly predicted. The positive predictive value was calculated at 73.52% based on the above data. However it is noteworthy that the type of cancer predicted on the nine samples was similar with that one mentioned in medical form. In Figure 1 are represented the above data.

As far as the types of abnormalities that were observed there was not specific pattern for each type of cancer. On Table 2 are summarized the most important and common abnormalities observed on specific types of cancer.

Table 1. Summarized results of patients as true positive and false positive. “Tested” refers to the outcome from aCGH experiments, while “Real” represents the data from patients’ medical forms.

| Tested | Real | Total |
|--------|------|-------|
| Positive (Cancer) |  |  |
| Positive (Cancer) | 32 | 2 | 34 |
| Negative (Healthy) | 1 | 8 | 9 |
| Total | 33 | 10 |  |

Table 2. The most common aberrations observed in aCGH experiments. The middle column referred to genes located on that locus and the final column represent the type of cancer correlated with each abnormality.

| Aberration | Genes | Type of cancer |
|-----------|-------|---------------|
| DEL 10q23.3 - q23.31 | KLLN | Breast |
| AMP 22q11.22 | MIR650 | Breast |
| AMP 21q11.1 - q21.2 | NCAM2 | Prostate |
| AMP 3q11.2 | EPHA6 | Prostate |
| DEL 3q12.2 | NIT2 | Colorectal |
| AMP 6q15 - q16.1 | FUT9 | Colorectal |
| AMP 11q25 | OPCML | Ovarian |
| AMP X p22.33 - p11.1 | GRPR | Gastrointestinal |
| AMP X q11.1 - q28 | EFN1 | Gastrointestinal |
| DEL 20q12 | PTPRT | Lung |
| DEL 16p13.3 | CREBP | Lung |
| DEL 17q25.1 | ACOX1 | Hepatocellular |
| DEL 20 q11.21 - q13.3 | - | Chronic myelogenous leukemia |
Figure 1. ROC curve analysis. The present figure represents the true positive rate (sensitivity) together with the false positive rate (1-specificity). The results show a specificity of 88.88% and sensitivity 94.11%. The confidence interval was set to 95% both for sensitivity and specificity.

4. Discussion

The determination of the primary origin of the tumor, as it has been mentioned, requires the examination of tissue; therefore biopsy is essential. Several experimental data in CUP demonstrated are characterized by chromosomal instability [10]. The existence of CTCs may be related with the metastatic ability of CUP as well as with other features, like sensitivity in therapy [11]. The identification of circulating tumor cells (CTCs), a population of cells derived from the primary tumor, gave a new impetus in biopsy, since all the examination require only a few milliliters of blood. CTCs arise from the primary tumor and throw through the blood stream, capable of creating new metastatic tumor [12]. The study of the above cells permitted scientists and physicians for timely and accurate results.

Conventional cytogenetic techniques of karyotyping and FISH (fluorescence in situ hybridization) are widely used to detect abnormalities. Chromosome analysis through karyotyping performed with culture and analysis of lymphocytes. Although scientists can observe the entire genome, the resolution is very limited. On the contrary, FISH has higher resolution than G-banding karyotyping and there is no requirement for specific stage at the cell cycle. The main disadvantage is that studied region is the one that is complementary to the probe [13] [14].

Comparative genomic hybridization (CGH) tried to fill the gap, but still the detection rate is not low enough, since it cannot identify small aberrations. Comparative genomic hybridization (CGH) is a cytogenetic assay used for detection of chromosomal abnormalities. It is an easy and quick method requiring only a few cells from the donor. Metaphase chromosomes are released from cells and they are hybridized with commercial slides, containing “control” chromo-
somes. The main disadvantage of this method is the detection rate, since it cannot identify abnormalities less than 3 - 5 Mb [15]. The array CGH, which is a combination of microarrays and CGH enables detection of smaller abnormalities, depending each time on the probes that are used [16]. Genetic abnormalities have been associated with different diseases including cancer. On this field, genomic aberrations might contribute to tumorigenesis and have been connected with the progression of the disease. Array CGH is widely used for prenatal and postnatal diagnosis of mental retardation, development problems, congenital malformation syndromes [17], but it can also be applied in human genetic studies [18]. In neonates has improved determination of anomalies with unknown etiology, where G-banding results could not be obtained [19]. Array CGH has been used for tumor classification and prediction of progression and prognosis [20] [21].

According to our experimental data, aCGH as a technique has the potential to discriminate healthy and cancer samples and furthermore to identify the primary origin of tumor with high sensitivity and specificity. Despite the fact that the size was not big enough, the data are encouraging and further experiments need to be performed in order to be used at clinical level.

**Conflicts of Interest**

The authors declare no conflicts of interest regarding the publication of this paper.

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