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Fundamental and Molecular Mechanisms of Mutagenesis

Genomic instability in silica- and cadmium chloride-transformed BALB/c-3T3 and tumor cell lines by random amplified polymorphic DNA analysis

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Abstract

Our earlier studies using random amplified polymorphic DNA (RAPD) analysis have shown genetic instability in human lung cancer tissues. Here we have investigated the potential for genetic instability in silica- and cadmium chloride ($CdCl_2$)-transformed BALB/c-3T3 cell lines. Non-transformed, transformed BALB/c-3T3 cells, and tumor cell lines (obtained by injecting nude mice with transformed cell lines) were analyzed for genomic changes. DNAs from 10 different transformed clones and their corresponding tumor cell lines were amplified individually by RAPD analysis using 10 arbitrary primers. DNA from non-transformed BALB/c-3T3 cells was used as a control to compare genetic alterations, if any, between non-transformed, transformed and tumor cell populations. PCR products from RAPD were electrophoretically separated on agarose gels and the banding profiles were visualized by ethidium bromide staining. Five of the 10 primers tested revealed genomic changes in silica-transformed cell lines when compared to non-transformed BALB/c-3T3 cells. Comparison of all 10 transformed and tumor cell lines showed varied degrees of genomic changes using all 10 primers. $CdCl_2$-transformed cell lines displayed fewer genomic changes, only three of 10 primers showed a positive result. $CdCl_2$-transformed cells and their corresponding tumor cell lines showed specific banding pattern differences in six of the 10 samples tested with six of the 10 primers. Changes in band intensity were the most commonly observed changes both in silica- and $CdCl_2$-transformed and tumor cell lines. The results seem to indicate a progressive change in genomic rearrangements which may directly or indirectly be associated with progression of tumorigenesis.

Keywords: Genetic instability; RAPD; Cell transformation; Tumorigenesis; Silica; Cadmium chloride; BALB/c-3T3 cell

1. Introduction

Genomic instability has been hypothesized to be a driving force behind the multistep process of

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carcinogenesis [1]. It is defined as an increase in the rate of genomic alterations, which can take many forms: point mutations, deletions, inversions, translocations, gene amplifications, and/or aneuploidy. Gene amplification has shown the most consistent correlation with tumorigenicity [2]. Multiple genetic changes are required for a normal cell to become tumorigenic [3]. If genomic instability increases the rate at which these alterations recur, then the accumulation of these changes and subsequent selection for growth and motility advantage may lead to the formation of neoplasia [4]. One successful strategy to study genomic instability has been to analyze abnormalities in cancer-related genes occurring between preneoplastic and neoplastic lesions in cell culture, animal models and humans. These gene abnormalities, such as mutations, can lead to genomic instability and aneuploidy, which are hallmarks of cancer [5]. Therefore, the role of genomic instability in carcinogenesis is being intensely investigated.

Random amplified polymorphic DNA (RAPD) analysis is a simple PCR-based technique to detect genetic instability [6,7]. In principle, it randomly amplifies DNA fragments using short arbitrary nucleotide primers. RAPD is a powerful tool for detecting genetic instability patterns and offers several advantages over other methods. A major advantage of RAPD analysis lies in the fact that it detects regions of amplifications, deletions and/or rearrangements without prior information about the loci. Another important feature of the RAPD method is that it permits simultaneous screening of multiple regions of the genome [8,9]. Furthermore, RAPD screening can be performed using a small sample amount and it is relatively easy to analyze the altered fragments. This technique has been used for DNA fingerprinting as well as for species classification and strain determination [6,7,10].

We have previously shown that DNA fingerprinting by RAPD allows identification of genetic alterations in human lung cancer tissues [11]. We have also shown that genetic changes in cancer tissues vary between samples. We have now applied the same principle to study progressive alterations among single cell clones obtained from silica- and $CdCl_2$-transformed and corresponding tumor cell lines. Using RAPD, we have compared the difference in banding patterns between the non-transformed BALB/c-3T3 cells, transformed clones induced by silica or $CdCl_2$ and tumor cell lines obtained by injecting the transformed cells into nude mice. We report the identification of genetic alterations in a progressive manner from non-transformed to transformed and finally, to tumor cells.

2. Materials and methods

2.1. Cell culture

BALB/c-3T3 clone A31-I-13 cells, kindly provided by Dr. E.J. Matthews (Hazleton Laboratories, Kensington, MD), were transformed either with 'Min-U-Sil 5' crystalline silica or 12 μM $CdCl_2$ using standard procedures [12]. Individual clones were selected and repopulated. Cells obtained from these transformed clones were injected into nude mice (2 million cells/axillary site). Tumors were formed within 2–4 weeks after injection. The tumors were aseptically removed and cultured for further analysis.

2.2. DNA extraction

DNA was extracted from approximately 10 million cells of non-transformed BALB/c-3T3 cells, transformed BALB/c-3T3 cells and tumor cell lines using the standard phenol/chloroform extraction and ethanol precipitation method [13]. Briefly, cells were incubated in 10 ml lysis/digestion buffer (1% sodium dodecyl sulfate, 1 mM EDTA, 50 mM Tris at pH 8.5, and 100 μg proteinase K per ml) at 52°C for approximately 16 h. The digested lysate was subject to two further extractions with equal volumes of phenol:chloroform:isoamyl alcohol (24:25:1). After centrifugation, DNA was precipitated from the aqueous phase by at least two volumes of cold absolute ethanol and collected with a glass rod [7]. The DNA was further purified by RNase digestion, two phenol/chloroform extractions, precipitated and collected as described above. The concentration of DNA was determined using a spectrophotometer.

2.3. RAPD analysis

A total of 10 arbitrary primers were used for RAPD analysis. The sequences of these primers are shown in Table 1. DNAs (0.5 μg) from each cell

Table 1
Primers used for RAPD analysis to detect alterations in non-transformed BALB/c-3T3, silica- and cadmium chloride-transformed and tumor cell lines

Serial number	Primer number	Sequence (5′–3′)
1	447	AACGGTCACG
2	448	TGGGCATCTG
3	449	GGTCTGAACC
4	450	ACGGTCTTGG
5	452	CCGGCTACGG
6	453	AGCTGCCGGG
7	457	AAGGCTAGCG
8	460	AGGCATTCCC
9	485	CGGCCCCTGT
10	488	CAGGCCCTTC

line (non-transformed and transformed BALB/c-3T3 cells and tumor cells) were amplified with each arbitrary primer (50 pmol), *Taq* DNA polymerase (1.25 U; Promega, Madison, WI), dNTPs (100 μM; Promega, Madison, WI) and $MgCl_2$ (2.5 mM) in 50 μl of reaction buffer for 40 cycles. PCR conditions were: denaturing at 94°C for 30 s, annealing at 40°C for 1 min and extension at 72°C for 1 min. PCR products (10 μl each) mixed with loading buffer were loaded on 2% agarose gels and electrophoresed with 100 V for 1 h. The gels were stained with ethidium bromide (0.5 μg/ml), visualized under UV light using an Eagle Eye II (Stratagene, La Jolla, CA) and photographed. The banding profiles were analyzed based on change in the intensity of a band, missing or appearance of a new band and compared between non-transformed and transformed and between transformed and tumor cell lines.

3. Results

A total of 10 pairs of transformed and tumor cell DNA samples were analyzed using 10 random primers. Non-transformed BALB/c-3T3 cell DNA from four passages (two different cultures from each passage) were analyzed simultaneously using all 10 different primers. The results of this study indicate that there were no changes in banding pattern between each of the non-transformed BALB/c-3T3 cell cultures. A representative gel using primer 488 is shown in Fig. 1. Genomic changes in both silica- and $CdCl_2$-induced transformed cell lines were detected by RAPD analysis (Tables 2 and 3). Banding

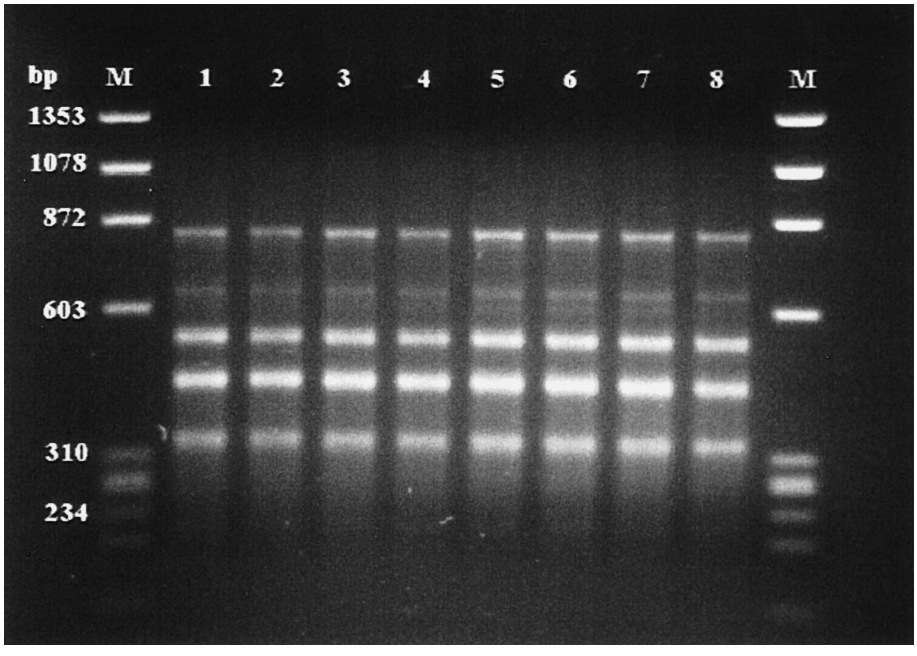

Fig. 1. Banding pattern of four passages (two different cultures from each passage) of non-transformed BALB/c-3T3 cell DNA using primer 488. M:øX174/*Hae*III molecular weight marker; lanes 1 and 2: DNA samples from passage 7; lanes 3 and 4: DNA samples from passage 8; lanes 5 and 6: DNA samples from passage 9; lanes 7 and 8: DNA samples from passage 10.

Table 2

(A) Genetic instability detected in transformed cell lines induced by silica when compared to non-transformed BALB/c-3T3 cells

Primer	Genetic alterations in transformed cell lines[a]									
	I	II	III	IV	V	VI	VII	VIII	IX	X
447	–	–	–	–	–	2	2	–	–	–
448	1	3	2	3	3	–	1	–	1	–
452	2	–	2	2	2	–	–	–	–	–
453	–	–	–	–	–	–	–	–	–	3
488	2	–	2,3	2	2	2	2	2	2	–

(B) Genetic instability detected in tumor cell lines when compared with transformed cell lines induced by silica

Primer	Genetic alterations in tumor cell lines[b]									
	I	II	III	IV	V	VI	VII	VIII	IX	X
447	–	2,3	3,2	1	–	2,3	1	–	–	–
448	–	1,3	2	–	–	–	–	–	3	–
449	–	–	2	–	–	–	–	–	–	3
450	–	3	–	–	–	–	–	–	–	–
452	–	2,3	1,2,3	–	–	–	–	–	–	2,3
453	–	–	1,2,3	–	–	–	–	–	–	1,2
457	–	–	–	3	3	3	–	3	–	–
460	3	–	–	–	3	–	–	–	–	–
485	–	–	–	–	–	2	3	–	–	–
488	3	–	1,2,3	–	1,3	–	–	–	–	–

1 = Missing band, 2 = new band appearance, 3 = change in intensity of the band.
[a]The genetic alterations shown here are from 10 transformed cell lines which were compared with non-transformed BALB/c-3T3 cells.
[b]The genetic alterations shown here are from 10 tumor cell lines which were compared with transformed cell lines.

Table 3

(A) Genetic instability detected in transformed cell lines induced by cadmium chloride when compared to non-transformed BALB/c-3T3 cells

Primer	Genetic alterations in transformed cell lines[a]									
	I	II	III	IV	V	VI	VII	VIII	IX	X
449	1	–	–	–	–	1	–	–	–	–
453	2	2	2	2	2	2	2	2	2	2
488	2	2	2	2	2	2	2	2	2	2

(B) Genetic instability detected in tumor cell lines when compared with transformed cell lines induced by cadmium chloride

Primer	Genetic alterations in tumor cell lines[b]									
	I	II	III	IV	V	VI	VII	VIII	IX	X
447	–	–	–	–	–	–	3	–	–	–
449	2	–	–	–	–	2	–	–	–	–
452	–	–	–	–	3	–	–	–	–	–
453	3	–	–	–	1	–	–	–	–	3
457	3	–	3	–	3	3	3	–	–	–
460	–	–	–	–	–	–	3	–	–	–

1 = Missing band, 2 = new band appearance, 3 = change in intensity of the band.
[a]The genetic alterations shown here are from 10 transformed cell lines which were compared with non-transformed BALB/c-3T3 cells.
[b]The genetic alterations shown here are from 10 tumor cell lines which were compared with transformed cell lines.

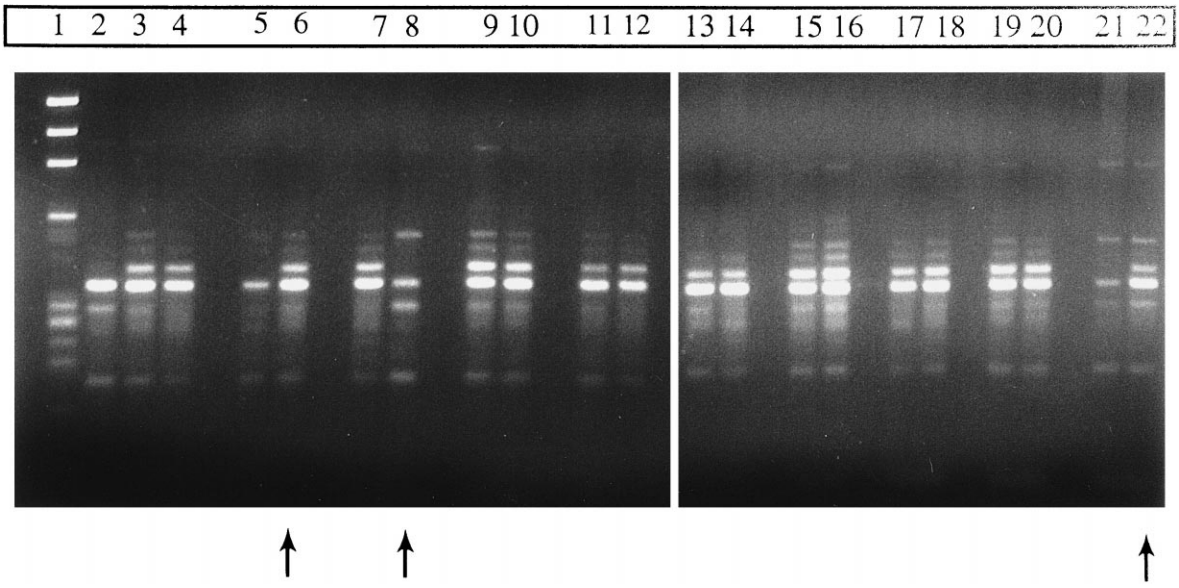

Fig. 2. Banding pattern of 10 pairs of Min-U-Sil 5 crystalline silica-transformed and tumor cell DNA using primer 452. Lane 1: øX174/*Hae*III molecular weight marker; lane 2: DNA from non-transformed BALB/c-3T3 cells; odd numbered lanes (3–21): DNA from silica-transformed cell lines; even numbered lanes (4–22): DNA from tumor cell lines. Note: lane 6 (arrow) shows addition of a new band; lane 8 (arrow) shows a deleted band and the appearance of a new band; lane 22 (arrow) shows a change in the intensity of a band.

pattern of 10 pairs of Min-U-Sil 5 crystalline silica-transformed and tumor cell DNA using primer #452 is shown in Fig. 2. Silica-induced transformed cell lines showed genomic instability when compared to non-transformed BALB/c-3T3 cells. Silica-transformed DNA samples demonstrated genomic instability in five of the 10 primers used when compared to non-transformed cells (Table 2A). Primer 488 detected changes in eight of 10 silica-transformed cell lines compared to non-transformed BALB/c-3T3 cells. The comparison showed that cell lines III, IV and V had the greatest number of changes detected by three different primers. Comparison of transformed and tumor cell lines also showed increased genetic alterations (Table 2B). The alterations exhibited in tumor DNA included the loss of a normal band (Fig. 3, panel 3), appearance of a new band (Fig. 3, panel 1), and change in the intensity of a pre-existing band (Fig. 3, panel 1). Many primers detected more than one such alteration in a given tumor cell line. Five of 10 cell lines showed changes as detected by primer 447 (Table 2B). Three of 10 cell lines had either changes in the intensity of band or missing/additional bands as detected by primer 448. Primers 449, 450, 460 and 485 showed minimal alterations in banding patterns. Only changes in the intensity of bands was observed using primers 457 and 460. Primers 447, 452, 453 and 488 showed the

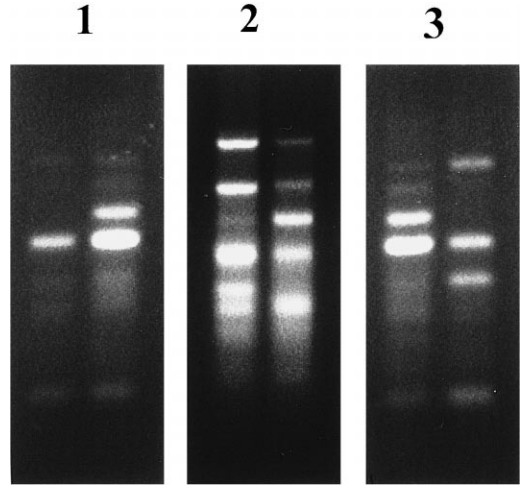

Fig. 3. Examples of banding pattern changes between transformed and tumor cell DNAs: (1) example of addition of a band, (2) example of change in band intensity and missing band, (3) example of missing band, addition of a new band and change in band intensity.

most changes in banding patterns. Cell lines II and III had the greatest number of changes detectable by several different primers. Percent detectability of primers (number of primers that could detect changes in 10 cell lines tested) for cell lines II and III were 40% and 60%, respectively.

The genome of $CdCl_2$-transformed cells appeared to be more stable than that of silica- transformed cell lines. Only three primers detected alterations in $CdCl_2$-transformed cell lines compared to non-transformed BALB/c-3T3 cells (Table 3A). All cell lines using primers 453 and 488 showed the appearance of a new band. However, more changes in the banding profiles were observed when $CdCl_2$-transformed and tumor cell lines were compared. Three out of 10 primers showed altered bands in cell lines I, V and VII (Table 3B). These results indicate that genetic instability may play an important role in the multistep process of silica- and cadmium chloride-induced tumorigenesis. Also, silica may have a greater potential to induce genomic instability when compared to cadmium chloride.

4. Discussion

Characterizing genetic alterations is an important initial step in our understanding of molecular mechanisms leading to the onset of tumorigenesis. Cell transformation may be a good starting point for the analysis of genetic alterations since carcinogenesis is a multistep process and cell transformation is thought to be an early step in occurrence. Strong evidence supports the concept that cancer is a genetic disease involving the clonal evolution of transformed cells [1,14]. Cancer cells arise through the accumulation of mutations either inherited or acquired in critical protooncogenes and tumor suppressor genes. Each mutation may provide an additional growth advantage to the transformed cells as they dominate their normal counterparts [3,15]. In addition to specific mutations, changes in DNA repeat sequences and additions or deletions of short regions are responsible for genetic alterations.

Cell transformation is a multistep process in carcinogenesis involving loss of growth control. It involves stages such as initiation, promotion and progression. Essentially, this is a process of cancer development. Cancer cells are generated from normal cells by accumulation of genetic alterations and may lead to accelerated tumor progression [1]. These alterations may be due to mutation, deletion, amplification or rearrangement in tumor-related genes [16,17]. Some mutations cause genetic instability and these multiple mutations exhibit a mutator phenotype [18]. This mutator phenotype could also be due to defect in the DNA repair gene, DNA replication, changes in cell cycle regulation, and apoptosis. The course of malignant transformation proceeds in a step-wise fashion and each incremental step up is caused by an additional genetic alteration.

When studying the progressive genomic changes, it would be useful to be able to directly and simultaneously non-transformed cell DNA, transformed cell DNA and tumor cell DNA from the same cell lineage. This will result in a better analysis of the samples and we may be able to predict the sequence of events that are occurring during the process of carcinogenesis. In this study, we have compared non-transformed, transformed and tumor cell DNAs simultaneously. The results of this study indicate that there are progressive changes from non-transformed to transformed cells and then from transformed to tumor cells, although the changes are not common to all the cell lines. Since none of the different non-transformed cells showed any alterations in banding patterns, the changes do seem to be significantly related to transformation. It is possible that the genome of a transformed cell is less stable than most non-transformed cells and that the genome of tumor cells are even less stable than the transformed cells. Also, silica seem to induce more genetic changes compared to cadmium chloride. Five of 10 primers showed changes in banding pattern when compared between non-transformed and transformed cells while only three of 10 primers showed changes in cadmium chloride-treated cells. Similarly, all 10 cell lines had changes using all 10 different primers when compared between silica-transformed and tumor cells, however only six cell lines showed changes in six primers when compared between cadmium chloride-transformed and tumor cells. This indicates that silica induces more genetic changes compared to cadmium chloride. Also, different types of genomic changes are seen in different clones/tumors because individual changes do not necessarily always follow the same sequence, either in terms of their occur-

rence or in terms of the time involved. Each tumor progresses in its own way, in its own time, although the average time for growth of most tumors from similar origin is the same [1]. Tumor progression is an example of an evolutionary process that is driven by an increased level of genomic instability or by an accumulation of genetic changes in the tumor cells and some degree of selective pressure in the growth environment of the tumor [1,19].

RAPD has become a valuable tool to detect genetic instability/alterations including DNA rearrangements, DNA addition, DNA deletion, and ploidy changes in cells [20,21]. Compared to other techniques, RAPD is much easier to perform and may detect DNA changes in the whole genome. Our earlier studies [11] have shown that RAPD can be used for the detection of genomic instability in lung cancer tissues. The technique uses only microgram quantities of samples and can be detected by simple ethidium bromide staining to analyze the genetic alterations. Also, the majority of the transformed cell lines showed changes with at least one of the primers and since no changes were observed between different non-transformed cell cultures, this seem to indicate that the changes are associated with transformation and RAPD is a useful technique for the detection of genetic instability.

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