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Detection of Genomic Alterations in Human Cervical Cancer by Two-Dimensional Gel Electrophoresis

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Abstract Two-dimensional gel electrophoresis was used to comprehensively scan the whole genome of 6 cervical intraepithelial neoplasia (CIN) lesions, 7 cervical squamous cell carcinomas, 1 cervical adenosquamous cell carcinoma, and 2 cervical adenocarcinomas for multiple genetic alterations, such as DNA amplification, chromosome deletion, loss of heterozygosity, and chromosome translocation, as compared with the paired normal tissues. DNA spot analysis of the genomic 2-dimensional gels was performed by a computer color overlay system and by spot recognition software allowing for objective spot comparison and quantitation. Nine spots were found to be amplified in the cervical carcinomas while two amplified spots were detected in the CIN III lesions. Fourteen DNA spots were either reduced in their intensity or absent in cervical carcinomas as compared to their normal paired tissues. Reduction of intensity in 6 spots was observed in the 5 CIN III lesions. These genetic alterations may represent changes in cancer genes that are associated with human cervical carcinogenesis. Further characterization of these alterations may be significant to the understanding of cervical tumorigenesis and to the development of biomarkers for clinical trials in cancer chemoprevention. J. Cell. Biochem. 25S:41–48. © 1997 Wiley-Liss, Inc.

Key words: two-dimensional gel electrophoresis; cervical cancer; genomic alterations; genomic scanning; chemoprevention

INTRODUCTION

Cervical cancer is one of the leading causes of cancer death in women [1,2]. There are three major types of cervical cancer: squamous cell carcinoma, adenocarcinoma, and adenosquamous carcinoma [3]. Preinvasive intraepithelial cervical lesions, also termed cervical intraepithelial neoplasia (CIN), are characterized by abnormal cellular maturation and proliferation, and atypical nuclei in the epithelium [3]. The CIN lesions themselves and genetic alterations found in CIN lesions may provide a suitable target for intervention with chemopreventive agents [4].

Human papillomavirus (HPV) infection has been associated with the pathogenesis of cervical cancer but other genetic alterations are required for the development of malignancy [5,6]. For example, activation of proto-oncogenes (*erb*-B, *myc*, *bcl*-1, *ras*) [7–11] and inactivation of tumor suppressor genes (*p53* and allelic loss at 3p, 11p, 17p) have been detected in human cervical carcinomas [12–19]. Most high grade cervical squamous cell cancers contain HPV types 16, 18, 31, or 33, suggesting that HPV plays a causal role in the induction of cervical cancer [5,6]. The genetic basis for this stems from the observation that the tumor suppressor gene, *p53*, can be inactivated by HPV E6 protein, and another tumor suppressor gene, *Rb*, can be inactivated by HPV E7 protein [20,21]. HPV alone, however, is not sufficient for the induction of invasive cervical carcinoma since only a few of the HPV infected patients develop cancer [22]. Increasing evidence indicates that other genetic alterations are required for the development of invasive cervical carcinoma [7–19].

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Increased *ras* p21 expression was detected in 18% of CIN I, 29% of CIN II, and >50% of CIN III lesions and invasive cervical carcinomas [23,24], indicating that *ras* activation is an early step in cervical carcinogenesis. In addition, several oncogenes are amplified in cervical cancers. For example, *erb*-B, *myc*, and *PRAD* 1 (cyclin D/*Bcl*-1) were frequently amplified in human cervical cancer and/or their cell lines [7–11]. In some cervical tumors, *Myc* L1, H-*ras*, *Sea*, and *Gli* are amplified [7]. Thus, activation of proto-oncogenes seems to play an important role in cervical tumorigenesis.

Mutations of the tumor suppressor gene *p53* is observed principally in HPV-negative cervical cancers since HPV E6 and E7 would inactivate *p53* and *Rb* in HPV-positive tumors [12–15,20,21]. In addition to the *p53* gene, loss of heterozygosity (LOH) of chromosomes 1, 3p, 11p, and 17q were also detected [16–19], suggesting that multiple putative tumor suppressor genes are involved in the progression of cervical cancer. Identification of these novel tumor suppressor genes and detection of additional genomic alterations in cervical cancer is significant for understanding the genetic basis of malignant transformation in the cervix.

In this study, a newly developed two-dimensional gel electrophoresis (2-DGE) assay was used to scan the entire genome for DNA alterations in CIN lesions and invasive cervical cancers. This method involves cleaving of high molecular weight DNA, radioactive labeling and separating DNA fragments by 2-DGE [25,26]. By comparing the 2-DGE profile of tumor tissues with their normal tissue counterparts, genetic alterations such as amplification, allelic loss, and hyper/hypomethylation can be detected.

MATERIALS AND METHODS

Sample Collection

Ten cervical carcinomas (7 squamous cell carcinomas, 2 adenocarcinomas and 1 adenosquamous carcinoma) and their normal surrounding tissues, plus 5 CIN III lesions were collected from the Medical College of Ohio, the University of Texas M.D. Anderson Cancer Center, and the Ohio State University. The histopathology of all lesions was assessed by board-certified pathologists in each institution. All of the tumor tissues used in this study and their histopathological diagnosis are listed in Table I.

DNA Isolation

Figure 1 shows the procedure for 2-DGE analysis. High molecular weight DNA is essential for high quality 2-DGE. The general strategy for the 2-DGE has been described previously [25]. DNA strand breakage is minimized by careful isolation, storage and manipulation of DNA in this procedure. Guided by histopathologic evaluation of thin tissue sections, the appropriate area of frozen thick tissue was removed from the slide and placed immediately in digestion buffer containing proteinase K. Following triple phenol extraction, DNA was precipitated with ethanol and dissolved in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). The quantity and purity of the DNA was measured in a spectrophotometer at wavelengths of 260 nm/280 nm and the quality of the DNA was checked by electrophoresis in an 0.8% agarose gel.

Not I Digestion

About 2–10 μg of DNA was digested in 100 μl *Not* I (New England Biolabs, Beverly, MA) digestion buffer (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 100 mM NaCl) containing 100 units of Not I for a minimum of 2 h at 37°C.

Not I Site Labeling

The sticky ends generated from NotI restriction digests were filled with [^{32}P]α-dGTP and [^{32}P]α-dCTP. The reaction buffer contained 40 mM Tris-HCl (pH 7.5), 20 mM $MgCl_2$, 50 mM NaCl, 10 mM DTT, 2 μl each of [^{32}P]α-dGTP (6000 Ci/mmol) and [^{32}P]α-dCTP (6000 Ci/mmol) and 20 units Sequenase (version 2.0, United States Biochemicals, Cleveland, OH). The reaction was allowed to proceed for 20 min at 37°C, and terminated by incubation at 65°C for 5 min.

EcoR V Digestion

DNA was then digested with *EcoR* V (New England Biolabs, Beverly, MA) for 2 h at 37°C. The reaction buffer contained 100 units of EcoR V, 50 mM Tris (pH 8.0), 10 mM $MgCl_2$ and 50 mM NaCl.

First-Dimensional (1st-D) Gel Electrophoresis

First-dimensional electrophoresis was carried out in a specially designed vertical apparatus which contained 5 mm (inner diameter) glass tubes connecting the upper and lower

TABLE I. Genetic Alterations Detected in Human Cervical Lesions by 2-DGE

		Patient No.														
Spot	Alterations	1 CIN III[a]	2 CIN III	3 CIN III	4 CIN III	5 CIN III	6 SCC	7 SCC	8 SCC	9 SCC	10 SCC	11 SCC	12 SCC	13 AdSC	14 AdC	15 AdC
1	Amplification	–[b]	–	2X	–	–	3X	3X	5X	2X	3X	3X	2X	3X	3X	5X
2	Amplification	2X	–	–	3X	2X	2X	2X	4X	3X	2X	2X	3X	2X	3X	3X
3	Amplification	–	–	–	–	–	8X	–	2X	–	–	–	–	–	–	–
4	Amplification	–	–	–	–	–	8X	–	–	–	–	–	–	–	–	–
5	Amplification	–	–	–	–	–	–	4X	–	–	–	–	–	–	–	–
6	Amplification	–	–	–	–	–	–	20X	–	–	–	–	–	–	–	–
7	Amplification	–	–	–	–	–	5X	–	–	–	–	–	–	–	–	–
8	Amplification	–	–	–	–	–	–	–	2X	–	–	2X	2X	–	–	–
9	Amplification	–	–	–	–	–	–	–	–	20X	–	–	–	–	–	–
10	Reduced spot	–	–	–	–	–	–80%	–60%	–90%	–50%	–50%	–60%	–	–50%	–85%	–60%
11	Reduced spot	–	–	–	–	–	–80%	–60%	–90%	–60%	–50%	–60%	–	–50%	–85%	–60%
12	Reduced spot	–	–	–	–	–	–60%	–50%	–80%	–50%	–50%	–50%	–50%	–	–60%	–
13	Reduced spot	–	–	–	–	–	–60%	–	–80%	–50%	–	–85%	–	–	–50%	–
14	Reduced spot	–	–	–	–	–	–50%	–60%	–60%	–50%	–50%	–50%	–	–	–50%	–
15	Loss	–	–	–	–	–	+	–	+	+	+	–	+	–	–	+
16	Loss	–	–	–	–	–	–	+	–	–	–	–	+	–	–	–
17	Loss	–	–	–	–	–	–	NI	+	–	–	–	–	NI	–	–
18	Loss	–	–	–	–	–	+	NI	+	–	–	–	–	NI	–	+
19	Loss	–	–	–	–	–	+	+	+	–	NI	+	–	NI	+	+
20	Loss	–	–	–	–	–	+	+	+	+	–	–	–	NI	–	NI
21	Loss	–	–	–	–	–	–	–	NI	+	+	+	+	NI	–	–
22	Loss	–	–	–	–	–	+	–	–	–	–	+	–	–	+	+
23	Loss	–	–	–	–	–	–	+	+	+	–	+	+	–	+	–
24	Loss	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–
25	Loss	+	+	–	–	+	–	–	–	–	–	–	–	–	–	–
26	Loss	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–
27	Loss	–	+	–	–	–	–	–	–	–	–	–	–	–	–	–
28	Loss	+	+	–	+	NI	–	–	–	–	–	–	–	–	–	–
29	Loss	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–

[a]CIN III, cervical intraepithelial neoplasia III; SCC, squamous cell carcinoma; AdSC, adenosquamous carcinoma; AdC, adenocarcinoma.
[b]3X, 3-fold increase; –80%, decreased by 80%; –, no spot amplification or loss; +, spot loss; NI, not informative.

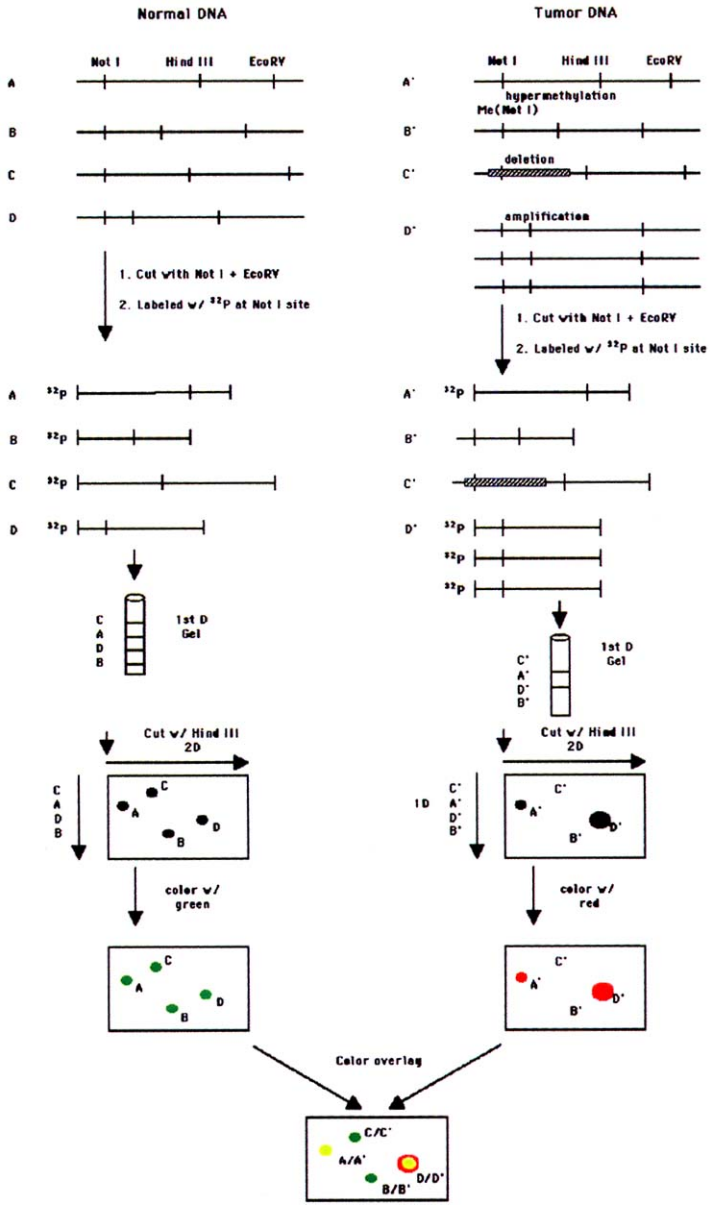

Fig. 1. The general procedure for 2-DGE analysis.

buffer tanks. The tubes (60 cm long) were filled with Seakem Gold agarose gel (FMC Bio product). The top 2 cm of the 1st-D gel piece (stacking gel) consisted of 0.5% agarose and 20% sucrose while the remainder of the gel (running gel) consisted of 0.8% agarose and 5% sucrose as previously described [25]. After *EcoR* V digestion, the DNA was loaded onto the top of the 1st-D gel and electrophoresed at 100 V for 2 h, followed by 230 V for 22 h.

In Situ Digestion With Hind III

The 1st-D gel was expelled from the glass tube and trimmed to a 30 cm long piece (so called "noodle") which contained most of the DNA. After 30 min equilibration in *Hind* III reaction buffer, the "noodle" was aspirated into a 6 mm inner diameter plastic tube and filled with *Hind* III reaction solution containing 2,000 units of *Hind* III. The tube was incubated at 37°C overnight.

Second-Dimensional (2nd-D) Gel Electrophoresis

After *Hind* III digestion, the "noodle" was equilibrated in 1× TBE buffer and loaded onto a 0.8% horizontal agarose gel, and electrophoresed at 45 V for 50 h. The agarose gel was then dried at 60°C and autoradiographed.

Scanning and Color Overlay System

A Bio-Rad scanner was used to convert the gel autoradiographs into digitized images with a spatial resolution of 400 μm. Analyses were performed on a Macintosh 840av computer using the public domain NIH Image program (written by Dr. Wayne Rasband at the U.S. National Institutes of Health and available from the Internet by anonymous ftp from zippy.nimh.nih.gov). A color hybridization Macro was used to register and align spot landmarks of normal and tumor tissues. One gel, usually the reference normal, was assigned a green look up table (LUT) and the other, usually the tumor, a red LUT. Gel images were then superimposed. The color of the two gels produced a profile of yellow spots at regions of similar spot intensity, green if the corresponding spot on the tumor gel was lower in intensity or absent and red if a novel or amplified spot was present on the tumor gel. The color shift, based on relative intensity of matched spots between the two gels, allowed a qualitative means for evaluation of spot intensity differences.

Scanning and Spot Detection

The 2-dimensional gel analysis software from Applied Imaging, Inc. was used for computer assisted analysis of relative spot density between reference normal tissue and tumor tissue gels.

Gel Matching

After monitoring and correcting the spot detection of all images, reference normal and tumor DNA images were matched. Automatic spot matching makes use of landmark references, usually ribosomal DNA, identified by the operator to "warp" spot coordinates within defined limits and identify matches between the two gels. Warping was a necessary analysis feature as gels set to run under "identical" electrophoretic conditions never yielded exact duplication over the entire surface. By iterative matching and landmark identification steps, all spots were identified as matched or novel fragments. After matching, the image file contained density information on every DNA spot for both normal and tumor gels for quantitative comparison.

RESULTS

A total of 7 squamous cell carcinomas, 2 adenocarcinomas, and 1 adenosquamous carcinoma were analyzed by 2-DGE for genomic alterations when compared to normal cervical tissues from the same patient. As shown in Figure 2, there are more than 3,000 spots in each of the 2-D profiles of either cervical carcinomas or their paired normal cervical tissues. After color-overlay analysis of each pair of 2-D gels, altered spots were identified and quantified by using computer-assisted systems. Figure 3 shows typical results from a color-overlay assay of a cervical cancer tissue vs. its paired normal cervical tissue. Upon screening, 9 spots were found amplified in 10 cervical carcinomas. As summarized in Table I, spot 1 was amplified 2–5-fold (100% of the cases); spot 2, 2–4-fold (100%); spot 3, 2–8-fold (20%); spot 4, 8-fold (10%); spot 5, 4-fold (10%); spot 6, 20-fold (10%); spot 7, 5-fold (10%); spot 8, 2-fold (30%); and spot 9, 20-fold (10%). Amplification of spots 3–9 was seen only in squamous cell carcinomas (patients 1–7). As shown in Table I, spot 1 was also amplified in 1 of the 6 CIN lesions analyzed and spot 2 in 3 of the 6 CIN lesions, suggesting that amplification of spots 1 and 2 may be early events in cervical carcinogenesis.

Five spots were found reduced in their intensity in cervical carcinomas. Spot 10 was reduced 50%–90% (90% of tumors), spot 11, 50%–90% (90%), spot 12, 50%–80% (80%), spot 13, 50%–85% (50%), and spot 14, 50%–60% (70%). The reduction in these spots may be attributed to either hypermethylation of DNA, or deletions and rearrangements of the genome in the tumor tissues [27–30].

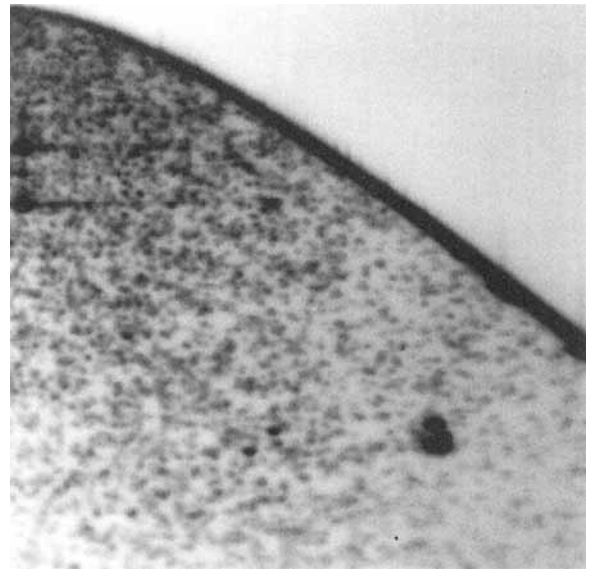

Fig. 2. A typical 2-DGE profile of human cervical tissue.

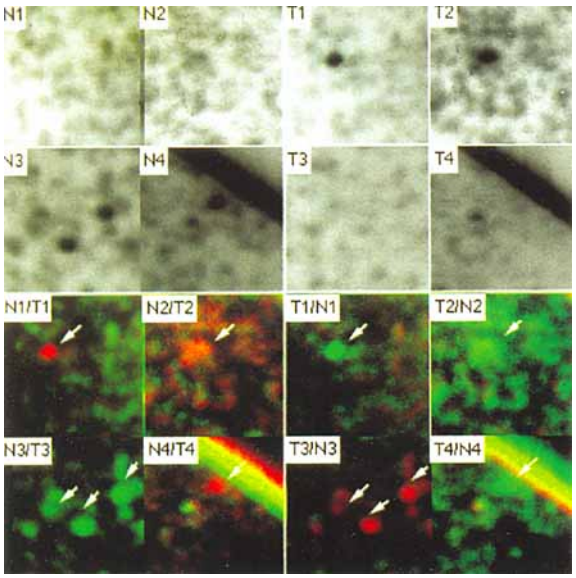

Fig. 3. A color-overlay analysis of the 2-DGE profiles of a human cervical carcinoma and its paired normal tissue. N, normal cervical tissue; T, cervical carcinoma; N1/T1, color-overlay normal tissue *(green)* vs. tumor tissue *(red)*; T1/N1, color-overlay of tumor tissue *(green)* and normal tissue *(red)*. N1/T1 and N2/T2 contain amplified spots, while N3/T3 and N4/T4 contain reduced spots.

Nine spots were completely lost in cervical cancer: spot 15 (60% of tumors), spot 16 (20%), spot 17 (13%), spot 18 (38%), spot 19 (75%), spot 20 (50%), spot 21 (50%), spot 22 (40%), and spot 23 (60%). Seven additional spots were lost in CIN lesions: spot 24 (60%), spot 25 (60%), spot 26 (100%), spot 27 (20%), spot 28 (75%), and spot 29 (40%). Hypermethylation or homozygous deletion may be responsible for the observed spot loss [27–30].

DISCUSSION

The results from this study, together with other recent reports [25–30], indicate that 2-DGE analysis of DNA is a very powerful and useful technique for detection of genomic alterations. This methodology can be used for high-speed survey for the presence or absence of restriction landmarks (or spots) throughout a genome and for measurement of their copy number in each locus, based on the principle that restriction endonuclease sites can be used as landmarks [25–30]. Since the total length of human genomic DNA is approximately 3×10^9 bp and around 3,000 spots can be revealed on one 2-DGE profile, 2-DGE can scan the restriction landmarks at intervals of less than 0.5 to 1 megabase (Mb). One spot on the 2-DGE profile corresponds to a single locus [26]. By using 2-DGE assisted by color-overlay analysis, we have shown that 9 spots were amplified in the cervical carcinomas and 2 were amplified in CIN lesions. In contrast, 5 spots were reduced in intensity and nine were lost in tumors when compared to normal paired tissues. The determination of the genes involved in these spots may provide insights into the mechanism(s) of cervical cancer development.

Some of the altered spots may represent genetic changes that have been observed previously. For example, gene amplifications previously detected in cervical cancer should be considered as primary candidates for the amplified spots found in the 2-DGE analysis. Amplification of HPV 16 and 18 were observed in some of the HPV-positive cervical carcinomas [5,6]. In addition, amplified proto-oncogenes such as *erb*-B, *myc*, *PRAD* 1, *Myc* L1, H-*ras*, *Sea*, and *Gli* were also detected in cervical carcinoma or their cell lines [7–11]. The loss of spots probably results from 1) homozygous deletions, since all lost spots were based upon a >50% reduction in intensity; 2) DNA rearrangements that could result in new spots and, simultaneously, the loss of spots in tumor tissues; and 3) hypermethylation, which could abolish the *Not* I site in tumor DNA when compared to paired normal tissue. Candidate genes for the lost spots could include the previously observed allelic loss of chromosomes 1, 3p, 11p, and 17q [12–19], and those tumor suppressor genes known to be inactivated by hypermethylation, i.e., *p16/CDKN2/MTS*1 and *VHL* genes [32–34].

The detection of DNA methylation that may inactivate a tumor suppressor gene(s) in tumor tissue is a major focus of cancer research [32–34]. Cytosine moieties at CpG sites in the mammalian genome are heavily methylated at the 5′-position [35]. Although the exact function of DNA methylation is unknown, current evidence suggests a role for DNA methylation in transcriptional control of gene expression [36]. There are approximately 6,000 *Not* I sites (*Not* I is a methylation-sensitive enzyme) per mammalian haploid genome and 90% of these are located in CpG islands [37] associated with transcriptional units [35,38]. In one 2-DGE gel, 3,000 spots can be seen that correspond to at least 1,500 different *Not* I sites (25% of total *Not* I sites). Thus, 2-DGE would appear to be the method of choice for analysis of methylation status in tumor tissues [39–42].

Some of the altered spots in cervical carcinomas and CIN lesions could be genetic markers that have not been previously identified. These novel changes can be further characterized by spot target cloning techniques [40,43–44]. The cloned DNA fragments will be used either as probes for Southern blot analysis to confirm the alterations, or as probes for screening cDNA libraries in order to determine whole cDNA sequences.

A major effort in cancer chemoprevention is to develop surrogate endpoint biomarkers for clinical trials in cancer chemoprevention [45]. 2-DGE analysis of preneoplastic and neoplastic lesions could provide new molecular biomarkers for the carcinogenesis process in specific organ sites. Furthermore, chemopreventive agent-specific biomarkers might be identified in tumor tissues treated with the agent. Recently, we used 2-DGE to scan genetic alterations in normal rat mammary tissues, and in methylnitrosourea (MNU)-, MNU/dehydroepiandrosterone-, and MNU/N-(4-hydroxyphenyl-) retinamide-induced rat mammary tumors [31]. More than 50 altered spots were seen in MNU-induced tumors while 20% to 30% fewer altered spots were observed in MNU/DHEA- and MNU/4-HPR-induced tumors [31]. Those spots suppressed by the two chemopreventive agents may be chemopreventive agent-specific markers in the rat mammary tumor model. Similarly, genomic alterations detected by 2-DGE in cervical lesions, especially those found in CIN III lesions including the amplifications of spot 1 & 2 and the losses of spot 24 to 29 (Table I), are strong candidates for surrogate endpoint biomarkers in human cervical cancer chemoprevention trials. Limitations of 2-DGE analysis of DNA include 1) no absolute correlation between altered spot in tumors and altered expression of mRNA or protein; and 2) this technique is more suited for the detection of genotypic changes rather than phenotypic changes.

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