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Original Research Report

Cervical chromosome 9 polysomy: Validation and use as a surrogate endpoint biomarker in a 4-HPR chemoprevention trial

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Abstract

Background. Several genetic alterations have been described in cervical cancers including: human papillomavirus (HPV) E6 and E7 oncoproteins, subtle sequence changes, alterations in chromosome number, chromosome translocations, and gene amplifications. This report focuses on establishing chromosome 9 polysomy as a cervical biomarker of chromosome instability and using it in a chemoprevention trial. Chromosomal instability is a feature of most human cancers and is probably an early event in the process.

Methods. We used 37 cervical cone specimens to validate chromosome 9 polysomy as a biomarker and then tested its modulation in a randomized clinical trial of 4-hydroxyphenylretinamide (4-HPR) in 39 patients with three blinded histopathologic reviews. No confounders were identified. In the present study, immunohistocytochemical analysis of Chromosome 9 polysomy was carried out and quantitatively measured.

Results. The Cell Index, the ratio of the number of total chromosome 9 copies to the total number of ells, increases significantly in archival samples as the cervix changes from normal to CIN to invasive cancer. In the chemoprevention trial, chromosome 9 polysomy was used as a biomarker and supported the histological analysis showing that 4-HPR impaired the natural regression response.

Conclusions. Chromosome 9 polysomy appears to be a marker of genetic instability that can be used in chemoprevention trials as a surrogate endpoint biomarker. In this randomized trial of 4-HPR, the chromosome 9 polysomy measurements supported the clinical histopathologic reading in a quantitative manner suggesting that 4-HPR at 200 mg/day may have been inhibiting the regression seen in the placebo arm by inducing genetic instability.

Keywords: Cervix; Randomized clinical trials; Chemoprevention; Surrogate end point biomarkers; Chromosome 9 polysomy

Introduction

The process of carcinogenesis is a multi-step process with many genotypic and phenotypic changes. Cancer is a genetic disease in which tumor suppressor genes, oncogenes, and stability genes play a role. Several genes must be defective in order for cancer to develop. Chromosomal instability, including extra copies of chromosomes, may be an early event in the carcinogenetic process [1–3]. Chromosome 9 polysomy has been used by our group in two ways: (1) as a biomarker of patients who are at increased risk of developing cancer, and (2) as a biomarker of response to interventions. One intervention of interest in oncology is chemoprevention. Chemoprevention agents are used to prevent or reverse the process of carcinogenesis. Clinical trials of chemoprevention agents use surrogate endpoint biomarkers to assess cancer risk and to monitor response to agents [4–6].

Cervical cancer is the second most common cancer worldwide and the survival rate for advanced disease is

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dismal. Cervical cancer is causally related to human papillomavirus (HPV) infection and the molecular events are now well understood. HPV E6 and E7 oncogenes integrated into the cellular DNA of the cervix drive the process of carcinogenesis by binding to Rb and p53. The immunobiology of HPV infections is less well understood; however, vaccines are currently being tested and have been highly successful in inducing antibody responses. Prophylactic vaccines, designed to induce an antibody response and prevent primary infections, have been studied and are successful in inducing increased antibodies. The length of protection remains unknown. Treatment vaccines, designed to induce T-cell recognition of HPV epitopes and to cause disease regression in those already exposed, have been less successful. The interplay of chromosome instability and immunobiology is not yet well understood and is a rich area for research. In summary, both cervical chemoprevention and immunoprevention trials need surrogate endpoint biomarkers.

While numerous studies have demonstrated genetic alterations in the cervix, none has examined chromosome 9 polysomy in archival samples. Relating these and other changes to HPV-induced carcinogenesis is critical. This study examines both chromosome 9 polysomy in archival specimens to validate the marker and its' use in a randomized placebo-controlled trial of 4-hydroxyphenylretinamide (4-HPR) in patients with high grade squamous intraepithelial lesions.

Materials and methods

Tissue materials

Archival specimens

Two hundred fifty archival cone specimens were carefully reviewed with the study pathologist and authors. Fifty-six cone biopsy specimens were selected for study. Study specimens were chosen so that the following categories were available for marker analysis: a set of normal tissues from normal patients, normal tissue beside areas of CIN, CIN1, CIN2, CIN3, CIS, and invasive sqaumous cancer. All cones were HPV typed with in situ hybridization. Table 1 shows the details of the archival specimens.

Clinical trial specimens

The clinical results of the placebo-controlled randomized clinical trial are reported elsewhere [7]. Quantitative pathology results are reported in by Yamal et al. [8]. Briefly, patients with high-grade squamous intraepithilial dysplasia, proved by colposcopically-directed biopsy, were consented and randomized to 4-HPR or placebo for 6 months. 4-HPR was given at 200 mg/day for 6 months with a 3-day drug holiday. Patients were followed at 3-month intervals with a colposcopy and Papanicolaou smear. The patients were evaluated for response at 6 months, followed for 6 more months, and underwent a final evaluation at 12 months. If a cervical lesion was present at the 12-month visit, a loop excision of the entire transformation zone of the cervix was

Table 1
Histopathology of archival cases

Case	Normal	Adjacent normal	CIN 1	CIN 2	CIN 3	CIS	Invasive cancer
1		x	x	x	x		
2				x	x		
3		x	x	x			
4		x	x	x	x		
5		x	x				
6			x				
7			x		x		
8		x	x				
9						x	
10		x	x	x			
11		x	x	x	x		
12		x	x				
13		x	x				
14		x	x				x
15							x
16							x
17			x		x	x	x
18			x				
19			x				
20			x				
21			x				
22							
23							x
24		x			x	x	
25		x			x		
26		x		x	x		
27		x			x		
28		x	x		x	x	
29		x				x	
30		x	x	x	x	x	
31						x	
32		x				x	x
33		x					x
34			x		x		
35		x	x	x	x		
36		x			x		
37		x			x		
38		x		x			
39		x			x	x	
40		x			x		
41		x			x		x
42		x	x		x		
43		x			x		
44			x	x	x		
45	x						
46	x						
47	x						
48	x						
49	x						
50	x						
51	x						
52	x						
53	x						
54	x						
55	x						
56	x						
Total	12	28	24	11	22	9	8

carried out, removing all the tissue at risk. If no lesion was present, then the patient was followed with continued colposcopies and Papanicolaou smears for 2 to 5 years.

Histological analysis

Archival specimens

Archival sections of Hematoxylin and Eosin (H&E) slides were reviewed with the study pathologist and all authors. The areas of interest were mapped with the pathologist and adjacent 4 μm sections were cut for chromosome polysomy analysis. Table 1 shows the details of the archival specimens.

Clinical trial specimens

Table 2 shows the details of patients in the clinical trial. A summary of the histopathologic responses is published in [8]. A blinded review of the H&E stained biopsies was carried out by the study pathologist and MF on the three separate occasions of the study biopsies. The principal investigator and pathologist were blinded to treatment arm and assigned the patients to 4 categories at the 6 and 12 month endpoints: Complete Response, Partial Response, No Change, and Progression. Complete Response was defined as no CIN in the loop excision specimen. Partial Response was defined as a regression to Cervical Intraepithelial Neoplasia (CIN) grades 1–2. No change was defined as the continued presence of CIN 2–3. Progression was defined as the progression from CIN2–3 to squamous Carcinoma in situ.

Chromosome in situ hybridization

Archival specimens

Tissue sections (4 μm) from paraffin blocks of biopsies were placed on silane-coated slides. The slides were placed overnight on a slide warmer at 65°C, then de-waxed in xylene for 15 min each, and cleared in 100% ethanol, two times for 10 min each. The slides were then incubated at 80°C for 1 h and then treated with 1 mg/ml Rnase in 2× SSC for 30 min. The tissue was then treated with 150–200 μg of 0.4% pepsin (Sigma Chemical Co., St. Louis, MO) in 0.2 N HCI as described previously [4,5]. Cover-slips were then placed. The slides were then placed at 4°C for 15 min to allow the pepsin to diffuse evenly throughout the tissue sections and then incubated at 37°C for 15–17 min to allow protein digestion. After three washes with deionized water for 3 min, each slide was dehydrated with alcohol using 70%, 90%, and 100% strength. The specimens were placed in acetone for 2 min at room temperature and air dried. Endogenous peroxidase activity was blocked by dipping the samples in 10% hydrogen peroxide in methanol for 5 min. Biotin-labeled α satellite DNA (0.8 ng/μl), specific for the centromeric region of chromosome 9 (Oncor, Gaithersburg, MD), was applied. The hybridization solution was composed of 60% formamide in 2× SSC, 5% dextran sulfate, 1 mg/ml salmon sperm DNA, and 0.8 μl biotinylated DNA probes. Thirty microliters of the hybridization solution was applied to each section and covered with a cover-slip and sealed with rubber cement. The probe and target DNA were denatured together at 95°C for 6 min and incubated at 37°C overnight. To detect the hybridized DNA, the cover-slips were removed and the slides were washed in 50% formamide, 1× SSC (pH 7.0) for 15 min twice at room temperature. The slides were then washed for 10 min in 0.1 SSC at 37°C. Avidine (100 μl of 5 μg/μl from Vector Laboratories, Incl., Burlingame, CA) in 3% bovine serum albumin in PBS was added and the slides were incubated at 37°C for 30 min in a moist chamber. The slides were then washed three times in PBD, anti-avidine in 3% bovine serum albumin was applied to the slides, and they were incubated for 30 min at 37°C. These steps were repeated twice to amplify the signals. The slides were then treated with avidine–biotin peroxidase complex solution (Vectastain ABC kit: Vector Laboratories Inc.). After being washed three times with PBD and PBS, the hybridized slides were stained with 50 ml of PBS solution containing 50 mg diaminobenzidine tetrahydrochloride (Sigma), 35 mg of $NiCl_2$, and 12 μl of 30% hydrogen peroxide at room temperature to allow signal development. The slides were then successively washed in 1× PBS for 5 min, rinsed in deionized running water for 10 min, air dried, and counter-stained with Giemsa stain for 30 s. The sections were mounted in Eukitt (Calibrated Instruments, Inc. Hawthorn, NY) and examined under a light microsope.

Clinical trial specimens

A slightly different procedure was carried out for CISH in the 4-HPR trial specimens. Rather than using the Oncor probe, for these studies, we used the DNA probe amplified from the human DNA clone pMR9A (GenBank accession number M64320) using two sets of primers (set 1: pEBR-25 and pEB9-170; set 2: pEB9-151 and pEB9-317) [8].

Analysis of chromosome signals

Archival and clinical trial specimens

Two indices of chromosome polysomy were identified. The Cell Index for each region was calculated by dividing the total number of signals detected by the total number of nuclei scored. The chromosome Polysomy Index was defined as the percentage of scored nuclei exhibiting three or more chromosome copies.

Table 2
Data from the randomized 4-HPR trial

Histological response	Progressive disease	No change	Partial response	Complete response	No return after baseline	Total
6 month						
4-HPR	2	13	4	1	2	22
Placebo	2	7	2	5	1	17

The levels of chromosome hybridization signals in the lymphocytes infiltrating the submucosa served as controls for determining the hybridization efficiencies in the epithelial layers. Chromosome copy numbers were assessed separately in the basal, parabasal, and superficial cell layers to determine whether the detected degree of genetic instability was influenced by cell maturation of location in the epithelium. From 200 to 900 cells were counted in each predetermined region at ×1000 magnification. The screening criteria included: (a) nuclei should not be covered by cytoplasmic materials, (b) nuclei should not overlap, (c) signal intensity should be more or less of the same homogenous staining intensity, (d) minor hybridization spots were not counted, (e) signals must be separated, and (f) paired or closely paired spots are counted as one signal.

The slides in the archival study were measured with the Magiscan Image Analysis system (Joyce Loebl, Ltd., Dukesway, England). This computer assisted imaging device was used to record the spatial distribution of chromosome counts in the epithelium. The relative *x*- and *y*-coordinates and detected chromosomes copy number of each scored nucleus were recorded in list mode. This permitted the preparation of a genetic map of the tissue section where each nucleus is represented by a dot, the color of which represents the chromosome copy number detected for that cell. The details of this method of representation have been published [4,5,8].

Results

Archival specimens

The chromosome Polysomy Index was defined as the percentage of scored nuclei exhibiting three or more chromosome copies. The mean and standard deviation of the Polysomy Index were plotted against diagnosis in Fig. 1. The number of nuclei ranged from 200–900 per specimen.

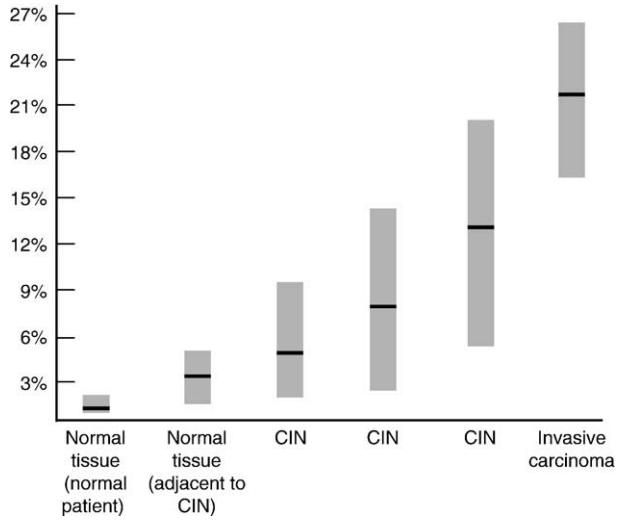

Fig. 1. Polysomy Index in archival samples of CIN and invasive cervical cancer.

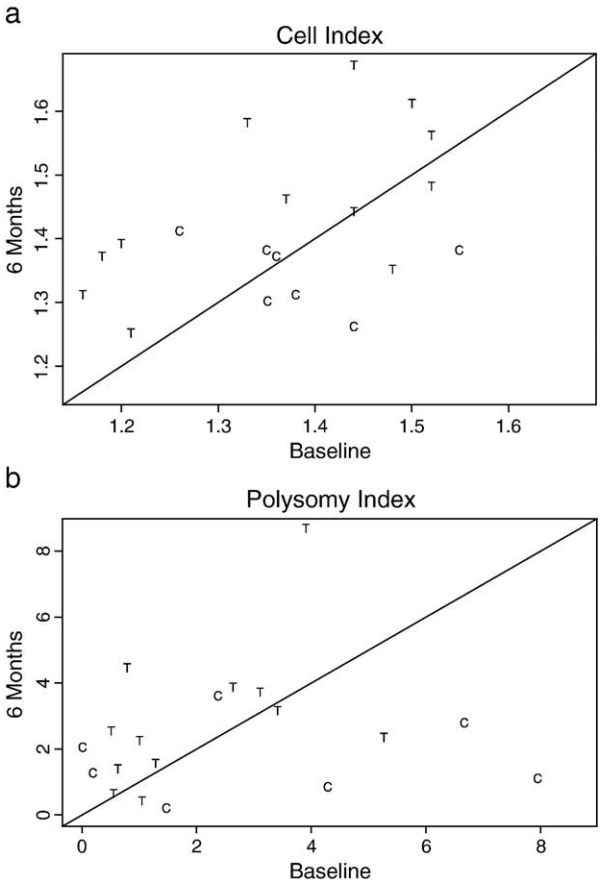

Fig. 2. (a) The figure shows a scatterplot of the Cell Index at baseline vs. 6 months after treatment with 4-HPR. The treated samples are labelled T and the placebo controls are labelled C. Both the axes are labelled in units of the Cell Index demonstrating the total number of chromosome 9 copies divided by the total number of cells counted per patient. The movement of treated patients to the upper left hand corner, that is to the higher Cell Index, demonstrates that the treatment either induced chromosomal 9 copies or inhibited their destruction. (b) The figure shows a scatterplot of the Polysomy Index at baseline vs. 6 months after treatment with 4-HPR. The treated samples are labelled T and the placebo controls are labelled C. Both the axes are labelled in units of the Polysomy Index which is defined as the percentage of scored nuclei exhibiting three or more copies. The movement of treated patients to the upper left hand corner, that is to the higher percent of Polysomy Index, demonstrates that the treatment either induced chromosomal 9 copies or inhibited their destruction.

Clinical trial specimens

Figs. 2 and 3 show results from the clinical trial specimens. The Cell Index was counted as the total number of copies of chromosome 9 copies divided by total number of cells counted per patient (average per cell). Most of the patients in the treated arm had Cell Index values which increased, while the control patients stayed

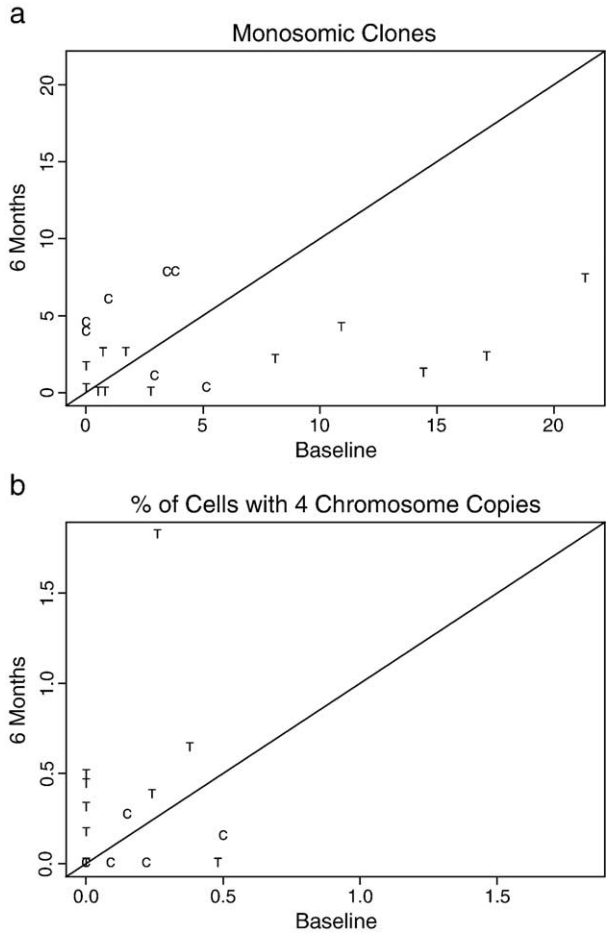

Fig. 3. (a) The figure shows a scatterplot of the count of chromosome 9 monosomy at baseline vs. 6 months after treatment with 4-HPR. The treated samples are labelled T and the placebo controls are labelled C. Both the axes are labelled in counts. This is a favorable finding. The lack of movement of treated patients to the upper left hand corner, that is to the higher count of monosomic clones after treatment, demonstrates that the treatment either induced chromosomal 9 copies or inhibited their destruction. (b) The figure shows a scatterplot of the percentage of four copies of chromosome 9 at baseline vs. 6 months after treatment with 4-HPR. The treated samples are labelled T and the placebo controls are labelled C. Both the axes are labelled in percent units, another type of the Polysomy Index, defined as the percentage of scored nuclei exhibiting four or more copies. The movement of treated patients to the upper left hand corner, that is to the higher percent of four copies, demonstrates that the treatment either induced chromosomal 9 copies or inhibited their destruction.

relatively the same or decreased on average. A Wilcoxon rank sum test gave a significant P value for this difference ($P = 0.025$).

The Polysomy Index and the percentage of cells with 3 or 4 copies of chromosome 9 per patient were calculated. The Polysomy Index also showed an increase in the value for the treated group, while the control group generally decreased.

Figs. 2a and b show scatter plots of the Cell Index and Polysomy Index for the patient samples. Again, the majority of the treated samples are in the left hand corner indicating a cancerous change. The Cell Index, the ratio of the number of total chromosome 9 copies to the total number of ells, increases significantly in 4-HPR arm over the 6 months of treatments ($P = 0.02$), as does the Polysomy Index ($P = 0.054$).

In Figs. 3a and b, we show the monosomic clones, a favorable factor, and the percentage of cells with 4 chromosome copies, an unfavorable factor. The percentage of monosomic clones decreases significantly in the 4-HPR arm, a sign that specimens fair more poorly ($P = 0.03$). While the percentage of cells with a triploid index is not statistically significantly different in this sample, those with greater than 4 copies of chromosome 9 are increased at 6 months in the 4-HPR arm ($P = 0.06$); like the Polysomy Index and the Cell Index, this confirms that the treated group faired more poorly than the placebo group. Because of the clinical histopathologic results, we suspended accrual to the trial. When these surrogate endpoint biomarkers confirmed that the biology of the lesions was unfavorable, we stopped the trial.

Conclusions

Surrogate endpoint biomarkers are important for chemo- and immunoprevention trials. Biomarkers need to be validated prior to use in trials. While the number of specimens for the validation in the archival group, was small, the findings are suggestive that chromosome 9 polysomy could be a useful biomarker. Since this biomarker decreased in the clinical trial compared to the natural or biopsy-induced regression in the control group and increased in the treated group, we believe the biomarker was modulated in the trial. Chromosome 9 polysomy has been better validated both as a risk factor and as a useful marker in clinical trials in the oral leukoplakia and laryngeal dysplasia [4–6].

4-Hydroxyphenylretinamide is a potent inhibitor of carcinogenesis in cell lines and animal models [9,10]. The mechanism of that action of 4-HPR is non-retinoic acid receptor mediated and involves the generation of radical oxygen species [11–14]. 4-HPR is considered a safe and well-tolerated retin compound and as such, is being studied in many Phase II trials in several organ sites [14]. Since five Phase I trials suggested that there may be ocular toxicity above 200 mg/day, this was set as the dose for the Phase II studies [14]. Veronesi reported no statistically significant differences in a 2972 women in a breast cancer prevention trial, followed for 97 months in a randomized trial of 4-HPR or follow-up. Post hoc analysis showed some potential benefit in premenopausal women [15,16]. Kurie et al. showed no statistically significant effect of 4-HPR in reversing squamous metaplasia in the lung [17]. Our group has demonstrated no significant regression and a possible negative effect 6 months after therapy [19].

Genetic alterations provide insights into tumor pathogenesis. Subtle changes, such as DNA methylation, have been demonstrated by Kim et al. [18]. Loss of heterozygosity has been demonstrated by several authors [19–21]. Gains in chromosome 3q have been described by Heselmeyer et al. [22]. Lengauer et al. review the genetic alterations in cancer; putting them into two broad categories: those for which the instability is due to subtle nucleotide alterations and those that are observed at the chromosomal level [23]. Chromosome 9 polysomy fits the latter category and has been validated in other cancers by our group [4–6].

Lengauer further states that the average colon, breast, pancreas, or prostate cancer has lost 25% of its alleles and it is not surprising to find a 50% loss in most tumors [23]. Losses or gains of chromosomes occur 10–100 times more frequently in cancerous cell lines compared to normal cell lines. The same results are found with regard to losses of heterozygosity. The high rates of gains and losses are consistent with a body of literature that shows that cancers are aneuploid, but that the karyotype of a single tumor may be heterogenous. Our group is interested in the interface between chromosomal instability and immunobiology of HPV-induced cervical carcinogenesis.

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