

Randomized Trial of Fenretinide in Superficial Bladder Cancer Using DNA Flow Cytometry as an Intermediate End Point¹

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Abstract

Retinoids have shown a potential activity in preventing tumor recurrence in superficial bladder cancer. We assessed the activity of the synthetic retinoid fenretinide in superficial bladder cancer using DNA flow cytometry and conventional cytology as surrogate biomarkers. A total of 99 subjects with resected superficial bladder cancer (pTa, pT1) were randomized to either fenretinide (200 mg day p.o. for 24 months) or no intervention. Cystoscopy and bladder washing for DNA flow cytometry end points (proportion of DNA aneuploid histograms, hyperdiploid fraction, and percentage of apoptotic cells) and proportion of abnormal cytological examinations were repeated every 4 months for up to 36 months. The primary study end point was the proportion of DNA aneuploid histograms after 12 months. This figure was 48.9% in the fenretinide arm and 41.9% in the control arm (odds ratio, 1.16; 95% confidence interval, 0.44–3.07). There was no difference in any other response biomarker between the two groups up to 36 months, nor was any biomarker able to predict recurrence risk. Recurrence-free survival was comparable between the arms (27 events in the fenretinide arm versus 21 in the control arm; $P = 0.36$). Twelve subjects in the fenretinide arm complained of diminished dark adaptability, and nine subjects in the fenretinide arm versus one control subject had mild dermatological alterations. We conclude

that fenretinide showed a lack of effect on the DNA content distribution and the morphology of urothelial cells obtained in serial bladder washings. Recurrence-free survival was comparable between groups. Because our data are hampered by the lack of predictivity of the selected biomarkers, additional studies are necessary to assess the activity of fenretinide in preventing bladder cancer.

Introduction

Bladder cancer is the sixth most common cancer in the United States, with approximately 54,000 new cases expected to be predicted in 1999 (1). Whereas the majority of cases initially present as superficial disease, and despite the efficacy of prophylactic BCG³ immunotherapy in prolonging recurrence and progression-free survival, a substantial proportion of cases are destined to recur within 5 years, including a 15–20% progression rate to muscle-invasive bladder cancer (2). Moreover, recurrences may occur anywhere in the urothelial lining, thus providing the rationale for preventive interventions aimed at counteracting the entire urothelial tract (2, 3).

Retinoids, the natural and synthetic analogues of vitamin A, have been used in the prophylaxis of recurrence of superficial bladder cancer (4–7). Whereas the results of these trials have been inconclusive, the toxicity of these agents, mainly etretinate and 13-*cis*-retinoic acid, was the major limiting factor (4–7). Fenretinide (*N*-4-hydroxyphenylretinamide) is a synthetic retinoid that is currently being investigated for the prevention of several solid tumors (8, 9). Importantly, the results of a Phase III secondary prevention trial in women with breast cancer show a distinct effect in pre- and postmenopausal women, with a 35% risk reduction of contralateral and ipsilateral second breast malignancies in premenopausal women undergoing fenretinide treatment, in contrast to a lack of efficacy and possibly an opposite trend in postmenopausal women (10).

In contrast to retinoic acid, fenretinide selectively induces apoptosis rather than differentiation in several tumor cell systems (11, 12) and maintains a stable plasma concentration during prolonged administration (13). Whereas its mechanism of action remains unclear, recent studies indicate that fenretinide may function through both receptor-dependent and -independent mechanisms (13–15). In preclinical studies, fenretinide was shown to reach high concentrations in the rodent bladder (16), to be the least toxic and the most active among several retinoids tested in mouse bladder carcinogenesis models (17, 18), and to induce apoptosis in bladder cancer cell lines at relevant pharmacological concentrations (19).

To increase the efficiency of chemoprevention clinical trials, much emphasis has been given to the search for surrogate

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³ The abbreviations used are: BCG, *Bacillus Calmette-Guérin*; PI, propidium iodide; DI, DNA index.

biomarkers (20, 21). Because genetic instability coupled with abnormal cell growth is an important component of carcinogenesis, and given the previous observation of the role of flow cytometric DNA content as a useful biomarker for monitoring treatment outcome (22), we selected DNA ploidy as well as proliferation and apoptosis, as measured by flow cytometry, and conventional cytology as potential intermediate biomarkers for a bladder cancer chemoprevention trial. In a previous pilot study (23), we showed the feasibility of monitoring fenretinide activity by measuring the flow cytometric DNA content in exfoliated cells obtained from serial bladder washings.

The aims of the present trial were: (a) to assess the role of the flow cytometric DNA content and conventional cytology as surrogate biomarkers of bladder carcinogenesis in epithelial cells obtained from serial bladder washings; and (b) to evaluate the activity of fenretinide in modulating the expression of these biomarkers. Because the study was not powered to assess a plausible effect of fenretinide on tumor recurrence (*i.e.*, a <55% risk reduction), the difference in recurrence-free survival between arms was recorded only for descriptive purposes. A preliminary analysis after 1 year revealed no significant difference in the primary end point (24). Moreover, in a recent study (25), we have shown that DNA flow cytometry and conventional cytology in cells obtained from bladder washings failed to qualify as suitable surrogate end point biomarkers during the 2-year treatment period. In the present work, we report the final results of fenretinide activity over the 3-year study period.

Materials and Methods

Study Design, Subjects, and Treatment. Subjects with a primary or recurrent superficial bladder cancer (stage Ta or T1, any grade) diagnosed within the previous 3 years were enrolled in three Urology Divisions in the city of Genoa, Italy. They were randomly assigned to oral fenretinide [R. W. Johnson Pharmaceutical Research Institute, Springhouse, PA; 200 mg/day, (two capsules at dinner)] or to no treatment for 2 years. Because fenretinide treatment is associated with a significant decrease in plasma retinol levels (26), a 3-day drug holiday was introduced at the end of each month to minimize diminished adaptation to darkness. The above-mentioned dosage and schedule were adopted based on the findings of a previous randomized dose ranging trial (27). A third year of follow-up was planned in both arms to evaluate any potential rebound effect. The study received Institutional Review Board approval, and all subjects signed an informed consent form before randomization. The limitation of toxicity evaluation without a placebo group was realized. The use of placebo was considered not advantageous due to the large size of the capsule (approximately 2.5×0.8 cm) and the notion that side effects were predictable based on previous studies. Moreover, rigorous procedures were adopted to preserve blinding while measuring treatment outcomes. Randomization was performed by telephoning the Central Coordinating Center. Patients were < 80 years; had performance status = 0 (WHO score); had normal liver, renal, cardiac, and metabolic functions; and had no contraindications to retinoid use. Moreover, they had to have no cystoscopic evidence of disease at the time of randomization and must have terminated intravesical treatment at least 2 months before randomization.

At baseline and every 4 months, all subjects underwent a complete medical examination and a cystoscopy with a bladder washing, using the technique described previously (24). Briefly, an irrigation was performed with five to six vigorous

pulses of 100 ml of normal saline through a urethral catheter or a resectoscope sheath. This procedure was repeated twice to increase cell availability. A total of 942 bladder washing specimens were analyzed. Blood hematology and biochemistry were repeated at 4-month intervals in the retinoid arm and every year in the control arm. The occurrence of adverse events was assessed by questioning the participant, and a specific questionnaire was adopted for visual disturbances (26). In case of a positive questionnaire, the patient underwent a complete ophthalmological consultation, including electroretinograms and dark adaptability tests, as described previously (26, 28). Any medical adverse event, whether related or not related to treatment, was recorded, and its severity was graded using the NCI toxicity criteria. Tumor recurrence was defined as the presence of a papillary tumor or an infiltrating cancer at the time of cystoscopy. The recurrent tumor was treated either by diathermy (if the maximum tumor diameter was < 5 mm) or by transurethral resection in all of the remaining cases. All patients undergoing transurethral resection subsequently received weekly intravesical BCG instillations (Pasteur strain; 75 mg for 6 weeks) or mitomycin C (40 mg for 8 weeks).

Compliance was evaluated primarily by capsule count. Circulating measurements of drug, its main metabolite 4-methoxyphenylretinamide, and retinol were also obtained after 1 year of treatment by high-performance liquid chromatography, using the methods previously described (13). Results of treatment compliance as measured by capsule count during the first intervention year indicated a compliance of 90% or higher in the vast majority of the patients (29).

Flow Cytometry. The collected fluid specimen of approximately 200 ml was immediately mixed and divided during the endoscopic session for cytology and flow cytometry, which were performed in two different laboratories of the same institution. To preserve blinding, each bottle containing fluid specimen was labeled by the clinical investigators with a progressive identification number only, so that neither the subject's identification nor the treatment allocation could be disclosed during the biomarker measurements.

Staining for flow cytometry and DNA measurements were performed as described previously (25, 30). Briefly, cells contained in 100 ml of fluid specimen were centrifuged, suspended in 0.5 ml of 0.5% paraformaldehyde for 15 min at 0°C, washed in PBS, permeabilized with 0.5 ml of 0.1% Triton X-100 for 5 min at 0°C, washed again with PBS, and finally suspended in the DNA staining solution (30 μ g/ml PI and 0.5 mg/ml RNase). After 1–2 h at room temperature, flow cytometric measurements were obtained using an EPICS Elite Coulter instrument (Coulter Corp., Miami, FL) equipped with suitable filters for PI excitation and emission fluorescence. For optimal cleaning from debris, the discriminator (trigger) was set on the photomultiplier for PI fluorescence. For each event, forward scatter, side scatter, and PI fluorescence signals (both area and peak for cell aggregate correction) were acquired and stored in list mode. A minimum of 10,000 cells were analyzed for each specimen.

The PI fluorescence mean channel of the lymphocyte population, which can easily be identified in the very large majority of bladder washings on the log FS-log SS bivariate plot, was used as the internal reference channel of diploidy. Aneuploid histograms were defined as those histograms with at least one discrete cell population with a DNA content other than diploid. The DI was evaluated as the ratio of the DNA content of G_0 - G_1 aneuploid cells to the DNA content of diploid G_0 - G_1 cells, which are conventionally given a DI of 1.00. Histograms

with multiple (n) aneuploid peaks were characterized by n DIs and n fractions of aneuploid cells. A histogram was considered aneuploid with $DI = 2.00$ when the tetraploid peak fraction exceeded by 2 SDs the mean value of the tetraploid peak fractions measured in 15 bladder washings from normal non-smoking donors, *i.e.*, 15% or higher. For a finer DI evaluation of aneuploid peaks that were very close to and often partially overlapping the diploid G_0 - G_1 peak, we used the bivariate log SS-lin PI fluorescence plot for easier identification. We operationally defined as being aneuploid those populations with a $DI \geq 1.10$. However, plots showing a slightly asymmetrical diploid population shifting to a higher PI fluorescence (PI fluorescence increase < 10%) were considered diploid. For all histograms, both diploid and aneuploid, the hyperdiploid fraction, a reliable index of cell proliferation (31), was calculated as the percentage of cells above the upper bound of the diploid G_0 - G_1 population. The S-phase fraction of diploid histograms and the aneuploid peak size were analyzed by using the Multicycle program (Phoenix Systems Software, La Jolla, CA). The percentage of apoptotic cells was calculated using the method described previously (32).

Because most bladder washing specimens contain a variable population of lymphocytes, monocytes/macrophages, granulocytes, and squamous cells in addition to the transitional epithelial cells, adjustment for the nontransitional cell component of the hyperdiploid fraction values was performed by computer-assisted image analysis of the cytological smears. The percentage of nonurothelial cells was calculated over 200 cells that were representative of the whole sample (CAS 200; Becton Dickinson). This was done under the assumption that nonurothelial cells are mainly quiescent and therefore enrich only the G_0 - G_1 cell fraction. DNA content histograms were not considered evaluable when the resolution of the measurement was poor, *i.e.*, when the coefficient of variation of the diploid G_0 - G_1 peak was higher than 6%, when the nontransitional fraction of cells was higher than 60%, or when the proportion of cell debris was excessive (>60%).

Cytology. Specimens for cytological examination were stained with the Papanicolaou technique and classified as one of four categories, according to the criteria proposed by Murphy (33), with slight modifications: (a) normal cells; (b) benign changes related to reactive cells (*e.g.*, following intravesical treatment or due to mild dysplasia); (c) low-grade neoplasia (atypical cells of uncertain significance, dysplastic cells, or suspicious abnormal cells); and (d) high-grade malignant cells.

Study End Points and Sample Size. The primary end point of the study was defined as the proportion of DNA aneuploid histograms after 12 months of treatment. Secondary end points were the proportion of DNA aneuploid histograms, the proportion of abnormal cytological examinations (class 3 and 4), the hyperdiploid fraction, and the percentage of apoptotic cells at yearly intervals for up to 36 months. Sample size was calculated assuming that approximately 70–80% of the patients had abnormal DNA content distribution at baseline and that treatment with fenretinide was associated with a 30% absolute decrease in the proportion of abnormal DNA histograms after 12 months (*i.e.*, from 70–80% to 40–50%). For an 80% power and a two-sided 5% significance, 90 subjects were necessary to detect such a difference. The number was increased to 99 assuming a 10% cumulative dropout rate at 12 months. Because the study was not designed for repeated measure analysis of the biomarkers or to detect a plausible difference in tumor recurrence as significant, the data of secondary end points were analyzed only for descriptive purposes.

Table 1 Main subject characteristics at baseline by treatment assignment

	Control ($n = 50$)	Fenretinide ($n = 49$)
Age (mean \pm SD; yrs)	61.6 \pm 9.5	63.8 \pm 8.6
Sex		
Male	41 (82.0) ^a	39 (76.9)
Female	9 (18.0)	10 (20.4)
BMI (mean \pm SD) ^b	25.3 \pm 3.5	26.4 \pm 3.8
Smoking habit		
Nonsmoker	15 (30.0)	8 (16.3)
Former smoker	14 (28.0)	24 (49.0)
Current smoker	21 (42.0)	17 (34.7)
Tumor history		
First event	17 (34.0)	19 (38.8)
Recurrent tumor	33 (66.0)	30 (61.2)
Recurrence index ^c		
Mean \pm SD	1.2 \pm 0.9	1.4 \pm 1.4
Median (range)	1.00 (0.2–3.6)	0.9 (0.2–8.3)
Stage ^d		
PTa	30 (60.0)	27 (55.1)
PT1	20 (40.0)	22 (44.9)
Grading ^d		
1	22 (44.0)	19 (38.8)
2	23 (46.0)	26 (53.1)
3	5 (10.0)	4 (8.1)
Previous intravesical treatment		
None	6 (12.0)	4 (8.2)
BCG	26 (52.0)	25 (51.0)
Chemotherapy	18 (36.0)	20 (40.8)

^a Numbers in parentheses are percentages.

^b BMI, body mass index.

^c Number of transurethral resections/person years.

^d Highest grade/stage is given for recurrent tumors.

Statistical Analyses. The association between treatment arm and DNA content measures or abnormal cytology was analyzed at each time, adjusting for baseline values by means of the Mantel-Haenszel test. The strength of this association was expressed by means of the odds ratio and 95% confidence interval. Differences in the hyperdiploid fraction between groups at each year were calculated by the Mann-Whitney U test. Actuarial recurrence-free survival was assessed with the Kaplan-Meier method and compared between the two groups by using the log-rank test (34). All of the recorded events were included in the analysis, regardless of treatment duration and compliance level, according to the intention to treat principle.

Results

Between September 1, 1993 and July 31, 1994, a total of 190 subjects were registered at the three urology units participating in the trial. Among these subjects, 65 (34%) proved to be noneligible (mainly due to the presence of tumor recurrence and also because of previous or concomitant medical diseases), whereas 26 (14%) refused to participate in the trial. A total of 99 subjects were randomized. The main characteristics of the study subjects are summarized in Table 1. Except for a lower proportion of nonsmokers in the fenretinide arm, all variables were evenly distributed between arms. Four subjects subsequently proved to be noneligible, two in the control arm (both had received systemic chemotherapy for their bladder cancer) and two in the fenretinide arm (one subject had a chronic hepatitis C and the other had received interstitial radiotherapy of the bladder). Moreover, one subject in the control arm was on intermittent treatment with etretinate for psoriasis. Because the results were unchanged when these five subjects were excluded, they were included in the present analysis. As of July

Table 2 Time course of flow cytometric DNA ploidy in serial bladder washings by treatment assignment

	N	DNA aneuploid		Odds ratio ^a (95% CI)
		n	(%)	
Baseline				
Control	45	25	(55.6)	
Fenretinide	48	34	(70.8)	
4 Months				
Control	46	36	(78.3)	
Fenretinide	43	30	(69.8)	
8 Months				
Control	47	26	(55.3)	
Fenretinide	42	23	(54.8)	
12 Months				
Control	43	18	(41.9)	
Fenretinide	47	23	(48.9)	1.16 (0.44–3.07)
16 Months				
Control	43	10	(23.3)	
Fenretinide	42	15	(35.7)	
20 Months				
Control	42	13	(31.0)	
Fenretinide	39	18	(46.2)	
24 Months				
Control	41	21	(51.2)	
Fenretinide	41	17	(41.5)	0.62 (0.21–1.77)
28 Months				
Control	38	15	(39.5)	
Fenretinide	42	21	(50.0)	
32 Months				
Control	32	17	(53.1)	
Fenretinide	38	20	(52.6)	
36 Months				
Control	36	16	(44.4)	
Fenretinide	41	23	(56.1)	1.69 (0.58–4.94)

^a Odds ratio and 95% confidence interval (CI) adjusted for baseline DNA ploidy.

31, 1997, when the study ended, a total of 82 subjects had completed the 3-year study period. Among the 17 subjects who did not complete the study, there were 5 deaths in the control arm *versus* 2 deaths in the fenretinide arm, whereas 3 subjects *versus* 2 subjects had adverse events, and 4 subjects *versus* 1 subject were lost to follow-up in the control group and the fenretinide group, respectively. In addition, seven subjects stopped fenretinide treatment prematurely but continued to be followed-up until the end of the study.

The time course of flow cytometric DNA ploidy in the cells obtained from bladder washings is shown in Table 2. No significant difference in the proportion of DNA aneuploid histograms was observed at 12 months or at any time for up to 36 months between the two groups. Likewise, no difference was shown in the degree of DNA aneuploidy; the median (and range) DI at baseline, 12, 24, and 36 months in the control group *versus* the fenretinide group was 1.12 (1.00–1.35) *versus* 1.15 (1.00–2.80), 1.00 (1.00–2.00) *versus* 1.00 (1.00–2.30; $P = 0.26$), 1.10 (1.00–2.00) *versus* 1.00 (1.00–2.32; $P = 0.18$), and 1.00 (1.00–4.00) *versus* 1.18 (1.00–1.34; $P = 0.47$), respectively. A similar pattern was observed on cell proliferation. Specifically, the median (and range) values of the hyperdiploid fraction at baseline, 12, 24, and 36 months in the control group *versus* the fenretinide group were 25 (5–77) *versus* 34 (2–86), 22 (6–89) *versus* 24 (6–81; $P = 0.77$), 10 (1–76) *versus* 10 (2–71; $P = 0.57$), and 10.5 (4–44) *versus* 13 (3–30; $P = 0.69$), respectively. No clear cut evidence of increased apoptosis was noted in any bladder washing, and the S-phase fraction of diploid histograms or the aneuploid peak size did not differ

Table 3 Time course of conventional cytology in serial bladder washings by treatment assignment

	N	Abnormal cytology		Odds ratio ^a (95% CI)
		n	(%)	
Baseline				
Control	50	6	(12.0)	
Fenretinide	48	5	(10.4)	
4 Months				
Control	48	9	(18.8)	
Fenretinide	46	16	(34.8)	
8 Months				
Control	47	9	(19.1)	
Fenretinide	45	10	(22.2)	
12 Months				
Control	46	12	(26.7)	
Fenretinide	48	9	(18.8)	0.66 (0.22–1.94)
16 Months				
Control	42	7	(16.7)	
Fenretinide	45	10	(22.2)	
20 Months				
Control	42	1	(2.4)	
Fenretinide	41	11	(26.8)	
24 Months				
Control	44	8	(18.2)	
Fenretinide	45	7	(15.6)	0.83 (0.23–2.93)
28 Months				
Control	38	5	(13.2)	
Fenretinide	43	7	(16.3)	
32 Months				
Control	33	4	(12.1)	
Fenretinide	38	3	(7.9)	
36 Months				
Control	38	3	(7.9)	
Fenretinide	44	6	(13.6)	2.20 (0.40–16.9)

^a Odds ratio and 95% confidence interval (CI) adjusted for baseline cytology.

between the arms (data not shown). Likewise, no difference between groups was observed in the proportion of abnormal cytological examinations in the cells obtained from bladder washings (Table 3).

Survival free of first tumor recurrence after the randomization is shown in Fig. 1. A total of 21 events were observed in the control group compared with 27 events in the fenretinide group ($P = 0.36$, log-rank test). Among these events, 11 recurrences in the control group and 12 recurrences in the fenretinide group were treated with diathermy. During the trial, 16 subjects in the control arm compared with 23 subjects in the fenretinide arm received intravesical treatment after tumor recurrence (13 subjects in the control arm and 19 subjects in the fenretinide arm) or for the presence of high-grade malignant cells in the cytological examination (3 subjects in the control arm and 4 subjects in the fenretinide arm). In total, 7 subjects in the control arm and 10 subjects in the fenretinide arm were treated with BCG, whereas the remaining subjects received mitomycin C.

Treatment compliance as evaluated by capsule count showed that 90% of the subjects had a 90% or higher treatment compliance; moreover, plasma fenretinide, 4-methoxyphenyl-retinamide, and retinol concentrations measured after 12 months were in the expected range, *i.e.*, 100–600 ng/ml (29). However, three subjects (9%) exhibited good compliance by capsule count but had no measurable plasma drug or metabolite levels (29).

Although limited by the lack of the placebo comparison, ophthalmological events were more frequent in the fenretinide

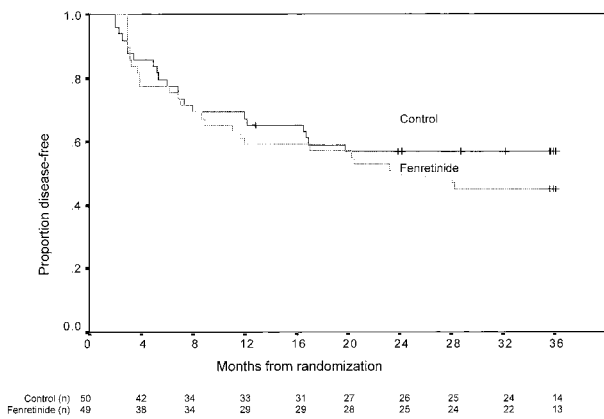


Fig. 1. Recurrence-free survival in the two treatment arms. There were 27 events in the fenretinide arm and 21 events in the control arm (observed/expected = 1.30; $P = 0.36$, log-rank test). The numbers at the bottom of the figure represent the number of subjects at risk for each time indicated.

arm, particularly diminished dark adaptation, which was fully reversible after treatment cessation (Fig. 2). In one subject, treatment had to be discontinued due to a claimed impediment in driving performance. Dermatological disorders were observed in one case in the control arm and in nine cases in the fenretinide arm ($P < 0.01$). These comprised four cases of pruritus, four cases of mucosal or skin dryness, and one case in each arm of skin desquamation. During the treatment period, there was no difference in the number of abnormal hematological and biochemical tests. Specifically, the percentage of grade 1 and 2 or higher alterations of the lipid profile (mostly an increase in triglycerides) at 12 months was 6.3% and 2.1% in the fenretinide group and 6.5% and 2.2% in the control group, respectively. At 24 months, these figures were 11.1% and 6.6% in the fenretinide group and 6.6% and 4.4% in the control group, respectively. Likewise, grade 1 and 2 or higher alterations of liver function were observed after 12 months in 10.4% and 2.1% of the subjects in the fenretinide arm and 2.1% and 0% of the control subjects, respectively. At 24 months, these percentages were 0% and 2.2% in the fenretinide group and 2.2% and 6.6% in the control group, respectively. Finally, the list of severe adverse events (*i.e.*, those events requiring hospitalization, life-threatening events, or events resulting in death) is shown in Table 4. No significant difference between arms was noted.

Discussion

The large costs of chemoprevention trials using cancer incidence as the main end point have generated efforts to devise intermediate trials using biomarkers as potential surrogate end points (21, 35, 36). A main objective of these trials is to provide important information for the implementation of larger trials aimed at assessing the effectiveness of experimental agents in reducing the incidence of invasive cancer in at-risk subjects. For patients with superficial bladder tumors, such a trial would require more than 1000 subjects to detect a remarkable 30% reduction in the rate of progression to muscle-invasive disease, which is the most appropriate clinical end point in this patient population (37).

The present study suggests that fenretinide is ineffective in modulating the flow cytometric DNA content distribution and the morphology of urothelial cells obtained in serial bladder

washings. Recurrence-free survival was not different between arms, although with a total of 48 events, the study had only sufficient power to detect differences that are not plausible (*i.e.*, >55% risk reduction). However, our results make it possible to rule out that fenretinide is associated with a 30% or greater reduction in the incidence of recurrences. Overall, the apparent lack of activity of fenretinide on cytometric and cytological end points as well as on tumor recurrence does not provide strong justification for a Phase III placebo-controlled trial for the prevention of muscle-invasive bladder cancer.

However, as discussed recently, our conclusion is limited by the observation that DNA flow cytometry and conventional cytology failed to qualify as reliable surrogate end point biomarkers of bladder cancer recurrence in a prospective and controlled setting due to their substantial variability over time (25). A poor intrasubject correlation was observed over the 2-year treatment time for DNA ploidy, hyperdiploid fraction, and cytology as well as between ploidy and cytology in each specimen. Moreover, aneuploidy was neither diagnostic of concomitant recurrence nor predictive of subsequent recurrence (25). Because our observation is based on a single institution trial, which enabled us to make centralized measurements of fresh specimens, interlaboratory variability, which turned out to be a major reason for poor intrasubject correlation for proliferation markers in other target tissues (38), cannot be advocated as a reason for the high variability of the biomarkers. Furthermore, the quality of our measurements was acceptable because only 5.1% of all specimens were not assessable due to a high proportion of debris or nonurothelial component, and the mean \pm SD coefficient of variation of the diploid peak of the lymphocyte population within each specimen was as low as $2.3 \pm 0.4\%$ (25).

Hence, our interpretation for the high variability of the study end points is, at least in part, intrinsically related to the DNA ploidy features of our "low-risk" patient population. Due to the early-stage bladder cancer of the patients enrolled in this study (pTa and pT1), approximately 80% of DNA aneuploid cell populations lies in the "near-diploid" region, with DIs ranging from 1.00–1.25 (25, 39, 40). Therefore, the compression of DI values in the narrow range of the diploid and near-diploid region might increase the risk of erroneous DNA ploidy estimations as a result of the experimental error variance. Studies have shown that this measurement variability may be as large as the slight difference between the diploid and the near-diploid DI values (31). Indeed, we have observed in a significant proportion of subjects a random stepping up and down of the DI between diploid and near-diploid values over time within the same subject. In addition, histograms classified as "DNA diploid" are "DNA flow diploid" only because the DNA flow cytometry technique is insensitive to early genetic events such as the gain or loss of small chromosomes or minor chromosomal deletions or translocation (41). Finally, inflammatory bladder specimens may also contribute to false near-diploid DNA histograms (42, 43), and nonspecific dye staining leading to increased DNA fluorescence has been described in large urothelial cells with large cytoplasm (31). For these reasons, our negative results with fenretinide must be interpreted with caution, and additional studies are advisable to assess the activity of this agent. Moreover, the slightly favorable trend on disease progressions and deaths in the fenretinide-treated group deserves further observation.

Seventeen subjects (12 controls and 5 treated subjects) dropped out of the study before completion of the 3-year period. Seven additional subjects withdrew from fenretinide treatment before completion of the 2-year treatment period but

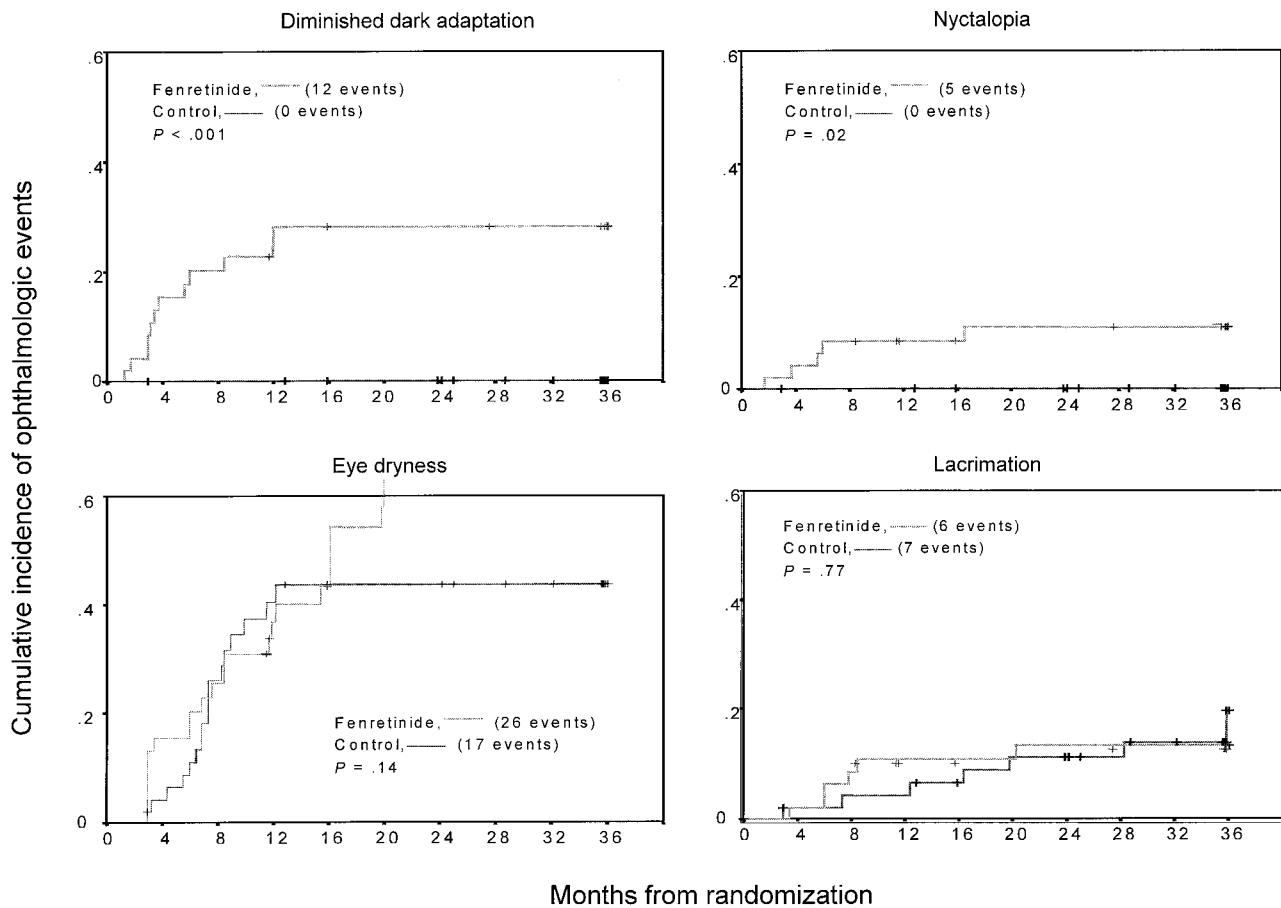


Fig. 2. Cumulative incidence of ophthalmological effects (n = number of subjects with event). Intervention ends at 24 months.

Table 4 Number of severe adverse events by treatment assignment

	Control	Fenretinide
Muscle-invasive bladder cancer	3 (1) ^a	1 (1)
Other malignancy	3 (2)	3 (1)
Gastrointestinal	3	2
Neurological	1 (1)	1
Infectious	1	2
Bone fractures	2	
Coronary heart disease	2	4 (1)
Stroke	2 (1)	1

^a Events resulting in death are shown in parentheses.

continued to be monitored with the biomarkers until the end of the study. Given the mean age of >60 years of the study population, this seems to be a reasonable drop-out rate. Among those subjects who completed at least 1 year of treatment, plasma fenretinide and metabolite concentrations fell within the expected range (100–600 ng/ml or 0.5–2 μ M; Ref. 29). Because a dose-response effect has been observed in the animal studies (17, 18, 44, 45), one possible explanation for the lack of effect of fenretinide may be due to failure to reach effective dose levels. In mice, fenretinide was effective at doses that are equivalent to four to eight times the corresponding human dose (17, 18, 44, 45). This contention is supported by the observation that no clear cut evidence of apoptotic cells was found by DNA

flow cytometry in most of our bladder washings. Indeed, fenretinide is known to be a potent apoptosis-inducing agent in several *in vitro* systems, including bladder cancer cell lines, at concentrations that are slightly higher than those achieved in the plasma of the patients at 200 mg/day (7, 11, 19). Fenretinide is known to concentrate at variable amounts in different target tissues, ranging from 5–10 times the plasma concentrations in the breast gland (46) to even lower than plasma levels in the prostate tissue (47). Given the easy accessibility of the organ, dose-response clinical studies using higher doses and appropriate biomarkers are advisable before further trials of fenretinide in bladder cancer prevention are implemented.

Despite the limitations in the evaluation of adverse events due to the lack of placebo, our study seems to confirm the good toxicity profile of fenretinide also in an elderly population. Diminished dark adaptation (25%) and dermatological alterations (20%) were the most common adverse events, but they were all reversible on treatment cessation. Treatment with fenretinide is associated with a reduction of circulating plasma retinol levels of approximately 70%, which accounts for the reduced rod sensitivity during dark adaptation (28). Although rarely troublesome, attention must be paid while driving because this effect may manifest itself when entering a tunnel on a sunny day and thus affect driving performance. Finally, our data confirm previous findings of a lack of negative effects of fenretinide on lipid profile and liver function, a remarkable finding compared with other retinoids (48).

In conclusion, fenretinide at 200 mg/day p.o. showed a lack of effect in modulating the DNA content distribution and on the morphology of urothelial cells obtained in serial bladder washings from subjects with superficial bladder cancer. Treatment with fenretinide was not associated with a dramatic reduction of recurrence-free survival. Because our conclusions are hampered by the poor stability of the biomarkers over time, additional studies are necessary to assess the ability of fenretinide to prevent bladder cancer in at-risk subjects.

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