

Assessment of Tumor Prevention in Type 1 Neurofibromatosis using a Nitroxide Compound

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ABSTRACT

Background: Type 1 Neurofibromatosis (NF1) is a genetic disorder linked to mutations of the *NF1* gene. Clinical symptoms are varied, but hallmark features of the disease include skin pigmentation anomalies (café au lait macules, skinfold freckling) and dermal neurofibromas. **Method:** These dermal manifestations of NF1 have previously been reported in a mouse model where *Nf1*^{-/-} mice are topically treated with dimethylbenz[*a*]anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA). We adopted this mouse model to test the protective effects of a nitroxide antioxidant, 5-carboxy-1,1,3,3-tetramethylisoinidolin-2-yloxy (CTMIO). Antioxidants have previously been shown to increase longevity in *nf1*-deficient fruitflies. Doses of 4μM and 40μM CTMIO provided *ad libitum* in drinking water were given to *Nf1*-deficient mice. **Results:** Consistent with previous reports, *Nf1*-deficient mice showed a 4.7-fold increase in papilloma formation (P < 0.036). However, neither dose of CTMIO had any significant effect on papilloma formation. A non-significant decrease in skin pigmentation abnormalities was seen with 4μM but not 40μM CTMIO. Subsequent analysis of genomic DNA isolated from papillomas indicated that DMBA/TPA induced tumors did not exhibit a local loss of heterozygosity (LOH) at the *Nf1* locus. **Conclusion:** These data reveal that oral antioxidant therapy with CTMIO does not reduce tumor formation in a multistage cancer model, but also that this model does not feature LOH for *Nf1*.

Key words: neurofibromatosis type 1, tumorigenesis, papillomas, antioxidant, nitroxide

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DOI: 10.5530/ax.2011.3.3

INTRODUCTION

Type 1 Neurofibromatosis (NF1) is an autosomal dominant genetic disorder that affects 1:3,000-1:3,500 individuals and can lead to disorders in a range of tissues.^[1] *NF1* has tumor-suppressor function and encodes for neurofibromin, a negative regulator of oncogenic protein Ras.^[2] A number of specific tumor types are associated with NF1, including cutaneous and subcutaneous neurofibromas, plexiform neurofibromas, astrocytomas, and optic gliomas. While the cutaneous neurofibromas are often benign, their appearance is common during puberty and they can be cosmetically disfiguring and can have a dramatic impact on patient quality of life. Currently there are no preventative measures able to suppress the onset of dermal neurofibromas in NF1 patients.

The causes of neurofibroma formation are linked to a clonal second hit on the NF1 gene.^[3] For plexiform neurofibromas, a *Krox20-cre; Nf1^{fllox/jlox}* mouse model showed that double inactivation of NF1 in Schwann cells was critical for tumorigenesis.^[4] However, this research also showed that interactions with *Nf1*^{+/-} mast cells were also important for potentiating tumor growth. A proportion of neurofibromas can progress to a more life-threatening outcome, a malignant peripheral nerve sheath tumor (MPNST). Coordinate loss of the master tumor suppressor *P53* may be associated with progression to an MPNST.^[5,6] The coincidence of tumor formation with puberty has historically led to speculation of an activation of tumorigenic progenitors by hormonal changes. This was recently investigated using a conditional inducible genetic mouse model. Topical application of tamoxifen on *CMV-CreER^{T2}*;

Nf1^{lox/-} mice was able to instigate recombination in skin-derived neural progenitors (SKPs) and give rise to dermal neurofibromas.^[7] Subdermal transplantation of *Nf1^{-/-}* SKPs had no effect in male mice, but gave rise to tumors in pregnant females, suggesting an interaction between *Nf1^{-/-}* progenitors and hormones.

While sophisticated inducible mouse models such as the one previously described reveal important insight into the biology of neurofibroma formation, tumor formation in patients is likely to be caused by sporadic double inactivation events due to DNA damage and ineffective repair. This has been previously modeled in NF1-deficient mice. Systematic and prolonged topical application of carcinogenic agents dimethylbenz[*a*]anthracene (DMBA) and 12-O-tetradecanoylphorbol-13-acetate (TPA) to the backs of mice has been shown to promote tumor formation.^[8] Using this system, *Nf1^{+/-}* mice treated with DMBA/TPA were found to have increased numbers of papillomas, as well as increased keratinocyte proliferation, skin pigmentation, and *H-ras* mutations in genomic DNA isolated from these papillomas.^[9] No tumors were observed in the wild type controls. We viewed this model as appropriate for the testing of therapeutic agents aimed at preventing DNA damage and subsequent tumor formation.

5-Carboxy-1,1,3,3-tetramethylisindolin-2-ylloxyl (CTMIO) is an antioxidant compound belonging to the nitroxide family of drugs. Antioxidants have produced beneficial effects in a *Drosophila* (fruitfly) model of NF1-deficiency. Administration of an anti-oxidant compound increased the longevity of *nf1*-deficient fruitflies and their resistance to oxidative stress.^[10] This led us to speculate that nitroxides may be able to reduce tumor formation in NF1-deficient mice and humans. CTMIO was an auspicious candidate compound as it was previously shown to rescue an ataxic mouse model, where oxidative stress leads to neural (nerve) degeneration.^[11] When administered to the ataxic mice, CTMIO was able to attenuate their neural degeneration.

In this paper we describe our application of CTMIO to the DMBA/TPA induced carcinogenesis model described

by Atit *et al.*^[9] CTMIO was provided to mice in their drinking water at doses that were known to be tolerated,^[11] and mice were pre-dosed with CTMIO for one month prior to exposure to carcinogens. TPA potentiation of tumors was applied for 7 months and outcome measures included papilloma number and histology, analysis of the *Nf1* gene in genomic DNA isolated from papillomas and skin pigmentation abnormalities.

METHODS

Animal ethics and husbandry

NF1-deficient mouse breeding and animal research experiments were approved by the SWAHS Animal Ethics Committee (protocols #5042, #1012). *Nf1^{+/-}* heterozygous knockout mice were a gift from L. Parada (UT Southwestern, Dallas, TX)^[12] and bred and maintained on a C57BL/6J background. Female mice were used exclusively and group housed in cages of up to 6 mice. *Nf1^{+/-}* heterozygous and *Nf1^{+/+}* control mice were generated from 25 breeding pairs set up simultaneously so that sufficient aged matched mice were available. Mice were given standard chow and autoclaved water *ad libitum*. Prior to and for one week following DMBA dosing, mice were placed on pelleted litter to minimize operator exposure to DMBA byproducts during cage changes; otherwise standard wood shavings were provided. Genotyped mice were assigned to the study and separated into groups (Table 1) at 2 months of age.

Tumor induction and nitroxide dosing

CTMIO (5-carboxy-1,1,3,3-tetramethylisindolin-2-ylloxyl) was synthesized according to the previously published procedure.^[13,14] Starting from 2 months of age, groups of mice were given solubilized CTMIO in their drinking water, and this treatment was continued until mice were culled. This drug was given at 4 μ M or 40 μ M concentrations, which were previously reported to be well tolerated by mice.^[11] Mice were visually monitored and weighed weekly throughout this pre-dosing phase.

Table 1. Experimental Design.

Group #	Genotype	Treatment	n =	Exclusions
1	<i>Nf1^{+/+}</i> (wt)	DMBA/TPA	15	4
2	<i>Nf1^{+/-}</i>	DMBA/TPA	15	0
3	<i>Nf1^{+/-}</i>	DMBA/TPA + 4 μ M CTMIO	15	2
4	<i>Nf1^{+/-}</i>	DMBA/TPA + 40 μ M CTMIO	15	0

At 3 months, tumor induction commenced using a combination of agents applied to a shaved region on the backs of the mice. A single dose of DMBA (40 μ g) was applied in 200 μ l of acetone. Subsequently, TPA (0.8 μ g) was applied in 200 μ l of acetone three times per week. Doses were applied by direct pipetting and mice were reshaved regularly. This regimen was applied for 6 months and tumors were allowed to continue growing for an additional month; mice were euthanased for end point analysis at 10 months of age. During this time, the mice were visually monitored daily for general appearance, condition of fur, activity, examined by handling 3 times/wk, and weighed weekly.

Outcome measures and statistical methods

At 10 months of age, all mice were euthanized for endpoint analysis including tumor and pigment incidence, and tissue harvest. At harvest, mice were shaved and papilloma growths of any size were scored by blinded technicians. Hyperpigmented patches of skin were similarly scored if they were greater than 1 cm in diameter. Exclusions for ethical/health reasons or due to anagen hair growth at the time of DMBA treatment were considered. Differences in tumor incidence were analysed by a one-tailed Fisher's Exact Test (<http://www.langsrud.com/fisher.htm>) to test the hypotheses that *Nf1*^{+/-} mice would show a higher tumor burden, and that CTMIO would reduce the tumor rate.

Papillomas were harvested for either DNA or haematoxylin and eosin staining. DNA was harvested using the Viagen DirectPCR Lysis Reagent supplemented with 0.3 mg/ml Proteinase K in an overnight incubation at 55 °C. DNA was amplified by PCR with primers detecting the presence of the *Nf1* knock-out and/or the wild-type allele.

Tissues harvested for histology were fixed in 4% paraformaldehyde overnight and then paraffin embedded. Sections were dewaxed, rehydrated, and stained with haematoxylin and eosin before dehydration and mounting.

RESULTS

DMBA/TPA induces papillomas and skin hyperpigmentation in *Nf1*^{+/-} mice

At three months of age, all mice were dosed with DMBA to induce tumorigenesis and started receiving TPA as a propagation agent 3 times per week. Tumor formation was regularly monitored as well as general mouse health and weight.

Some mice were seen to develop regions of hyperpigmentation (Figures 1A-B), which have been previously described for this model.^[9] Papillomas began to appear after approximately 3 months (mice aged 6 months), and some tumors were particularly large (Figure 1C). At the site of papilloma progression, we observed drastic epidermal hyperplasia with continued TPA treatment compared to normal skin. One mouse with a particularly sizeable and fast-growing tumor was prematurely euthanased for health reasons. One mouse developed an adverse inflammatory skin reaction suspected to be caused by overgrooming and exacerbated by the acetone vehicle. This mouse was also euthanased and excluded. Mice in the anagen phase hair growth at the time of DMBA treatment were excluded due to their sensitivity to the treatment as per the original description of the model system [9]. In total, six mice were excluded of the sixty mice assigned to the study.

Histological staining of papillomas showed an increased number of hair follicles in the dermal layer when compared to control skin (Figures 1D-E). The dermal layer was also thickened, indicative of significant hyperplasia.

CTMIO and tumor prevention

Mice in groups 3 and 4 were pre-treated with CTMIO for one month prior to tumor initiation with DMBA and subsequent propagation with TPA. At the experimental endpoint, tumor numbers, tumor rates, and patches of skin hyperpigmentation were counted for all groups.

In the control group 1/11 mice developed a single papilloma. No papillomas had previously been reported in control mice at this DMBA/TPA dose^[9]. In the *Nf1*^{+/-} control group that did not receive CTMIO, 6/15 mice developed a total of 9 papillomas. In the *Nf1*^{+/-} groups that were dosed with 4 μ M and 40 μ M CTMIO, 5/13 and 7/15 mice developed 6 and 8 papillomas respectively. Taken together, the *Nf1*^{+/-} mice showed a statistically significant 4.7-fold increase in tumor incidence ($P < 0.036$). Intra-group comparison of the *Nf1*^{+/-} mice dosed with CTMIO showed no significant decrease in tumor incidence with 4 μ M or 40 μ M CTMIO ($P < 0.62$, $P < 0.76$) (Figure 2A).

Hyper-pigmented patches were observed in all treatment groups. No significant differences was observed between wild type and *Nf1*^{+/-} control groups ($P < 0.25$) and while the low dose CTMIO decreased pigmentation, higher numbers of patches were seen in the high dose CTMIO group. However, none of these differences were statistically significant.

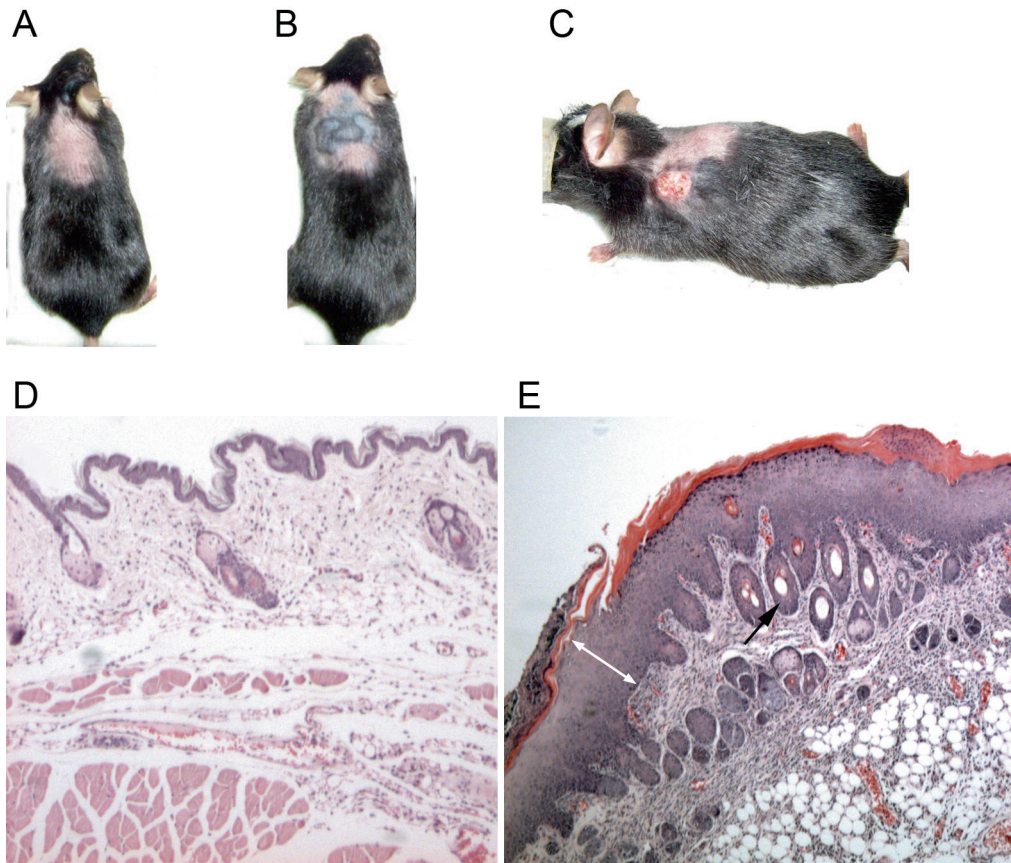


Figure 1. DMBA/TPA treatment induces papillomas and pigmentation patches in NF1 and control mice. Mice exposed to DMBA and TPA for six months were photographed to illustrate no reaction (A), regions of skin hyperpigmentation (B), and papilloma formation (C). Representative images of a control skin (D) and a papilloma (E) clearly indicates epidermal hyperplasia induced by TPA treatment (white arrowheads) and an increased number of hair follicles in the dermis (black arrow). The Nf1^{+/-} mouse groups showed significantly more papillomas than the wild type control group (*P < 0.036).

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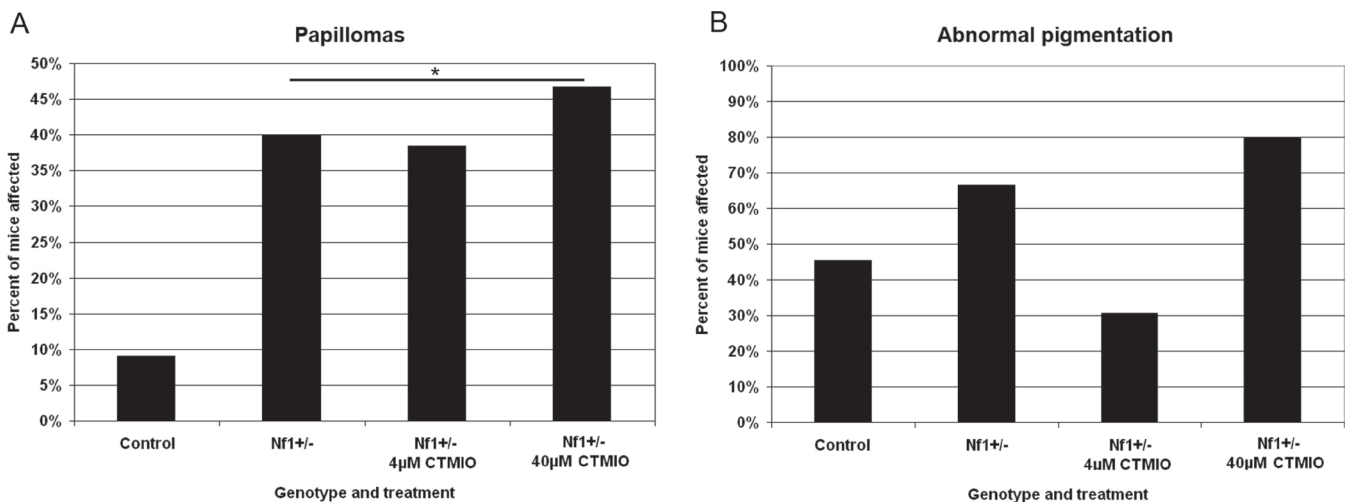


Figure 2. CTMIO treatment and papilloma development. Mice exposed to DMBA and TPA for six months (followed by one month of no exposure) were analyzed for papilloma formation (A) and the presence of skin pigmentation patches (B). Control mice are wild-type and did not receive CTMIO. The other 3 groups were composed of Nf1^{+/-} mice that received no CTMIO (Nf1^{+/-}), low dose 4µM CTMIO in drinking water (Nf1^{+/-}, 4µM), or high dose 40µM CTMIO in drinking water (Nf1^{+/-}, 40µM).

Papillomas are not associated with loss of heterozygosity

In NF1, neurofibromas are associated with a second somatic mutation in the *NF1* gene^[3]. The DMBA insult used in this study induces DNA damage by chemical adducts that covalently bind to DNA, and then progress to tumors by chronic exposure to TPA. While this has been reported to lead to mutations in *H-ras*,^[9] the status of the *Nf1* gene in papillomas was unclear. We speculated that the mechanism of tumor prevention by CTMIO would be to prevent a second hit to NF1, so it was essential to determine whether DMBA/TPA treatment was affecting this locus.

Genomic DNA extracted from papillomas was tested using primers specific for exon 31 of the murine *Nf1* gene. In a multiplex reaction, a second set of primers specific for the neoTK cassette in the transgene were used. In the *Nf1*^{+/-} mouse line, all non-tumor tail tissue was found to be heterozygous for the *Nf1* gene (data not shown). In DMBA/TPA induced tumors, exon 31 was found to be present, indicating that a loss of heterozygosity had not taken place, nor any microdeletions overlapping with exon 31 (Figure 3).

DISCUSSION

In this study we used a previously published multistage model of skin carcinogenesis in NF1-deficient mice^[9] to test the efficacy of an orally administered antioxidant compound. The compound CTMIO had been previously found to be effective in preventing degeneration caused by oxidative stress in an ataxic mouse model, using an analogous oral dosing regimen.^[11] If effective, such a protocol would represent a novel approach to preventing the numerous benign but disfiguring neurofibromas characteristic of NF1.

Previously, a two-stage DMBA/TPA induction has been found to generate papillomas at a high rate in *Nf1*^{+/-} mice (75%, 9/12 mice)^[9]. In terms of our study design, a power calculation showed that a similar group size would allow us to determine a 3-fold reduction in tumor prevalence; a value that was defined as clinically significant. We bred sufficient mice in order to assign initial group sizes of $n = 15$ to allow for exclusions due to hair in anagen phase growth at the time of DMBA dosing. Mice go through episodes of rapid hair growth during which they are more susceptible to cancer induction.^[15]

Our results are in agreement with Atit *et al.* in showing an increased papilloma rate in *Nf1*^{+/-} mice. Nevertheless, the overall papilloma rate was less than previously reported,

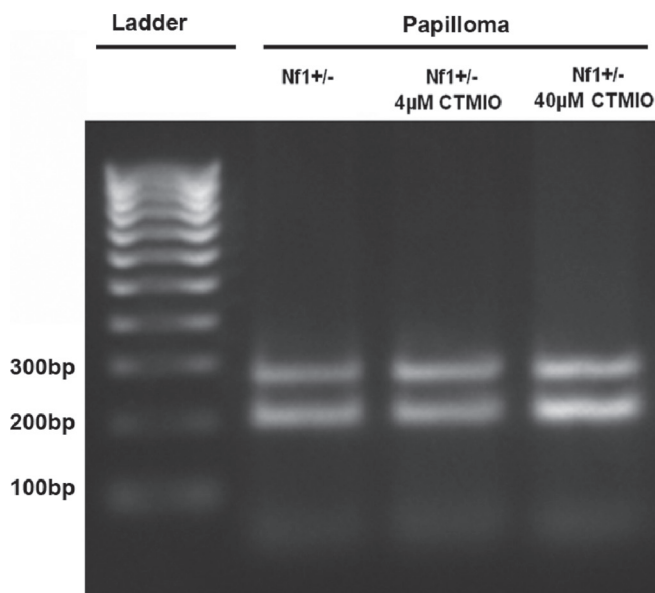


Figure 3. Loss of heterozygosity is not observed in papillomas. Genomic DNA was harvested from all three *Nf1*^{+/-} groups in our study (see Table 1) and PCR was used to determine whether the wild-type allele (280 bp) was lost. In all tumor specimens, no loss of heterozygosity was observed.

which could be attributed to several factors. Mouse strain can have profound effects on tumor rates in cancer models.^[8,16] Both were performed in a C57BL6 strain normally less susceptible to cancer induction,^[17] but there may be subtle geographical/temporal differences in the source colonies that could influence tumor susceptibility. To reduce mouse stress, our mice were group housed females compared to singly caged males used by Atit *et al.* However, sex-related differences in endocrine/hormonal activities could affect tumorigenesis as has been previously reported in another distinct NF1 mouse model.^[7] Additionally, there is potential for subtle experimenter-dependent differences in skin patch size and drug application protocols to influence tumorigenesis.

The primary measured outcome for this study was the papilloma rate, and we observed that neither CTMIO dose reduced the papilloma rate in *Nf1*^{+/-} mice. Furthermore, when all *Nf1*^{+/-} mice were pooled, irrespective of CTMIO dosing, a difference was still seen compared to control *Nf1*^{+/+} mice ($P < 0.07$). This suggests that antioxidant therapy with this nitroxide is ineffective in reducing the high tumor rate seen in an NF1-deficient multistage skin cancer model. Similarly, CTMIO had no significant impact on patches of skin hyperpigmentation as a secondary outcome measure.

NF1 tumors are associated with a double inactivation event which can be a second independent somatic mutation, or a loss of heterozygosity (LOH). The mechanism of LOH is poorly understood. LOH for NF1 has also been

reported at a rate of 20% in sporadic colon cancer.^[18] In an analysis of plexiform neurofibromas, LOH featured in 30% of tumors; in contrast, no somatic microdeletions were seen.^[19] A larger study reported LOH rates of 13% in dermal neurofibromas, 40% in plexiform neurofibromas, and 60% in malignant peripheral nerve sheath tumors (MPNSTs).^[20] In the prior DMBA/TPA-induced papilloma model Atit *et al.* the authors demonstrated spontaneous mutations in *H-Ras* that are associated with the multistage carcinoma model,^[8,9] but did not test for loss of heterozygosity (LOH) for *Nf1*. An analysis of genomic DNA extracted from all tumors did not show any LOH events or any large microdeletions corresponding with exon 31. This suggests that the NF1 state potentiates tumor formation by alternative mechanisms. Other somatic inactivating point mutations could also be present but remain undetected by LOH assays. Alternatively, as a negative regulator of Ras, NF1 haploinsufficiency could increase the effects of *H-Ras* mutations. Other NF1-deficient cells such as mast cells could also affect papilloma growth, as recently demonstrated for plexiform neurofibromas.^[21]

In conclusion, these data show that CTMIO is not effective in preventing papilloma formation in a NF1-deficient mouse model of skin carcinogenesis. However, there still remains the potential for this and related therapies to be effective in other models of NF1 where chemical or oxidative stress specifically leads to a LOH for NF1.

LIST OF ABBREVIATIONS

NF1	Neurofibromatosis, Type 1
DMBA	dimethylbenz[<i>a</i>]anthracene
TPA	12-O-tetradecanoylphorbol-13-acetate
CTMIO	5-carboxy-1,1,3,3-tetramethylisoindolin-2-yloxy
LOH	loss of heterozygosity

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