Research Report

The FHIT genome caretaker: a cancer prevention target

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ABSTRACT

FHIT encompasses the fragile site FRA3B on chromosome 3 which can be lost and cause genome-wide DNA instability. This instability can cause the loss or mutation of other caretaker genes such as BRAC1/2 genes which causes a specific mutational signature. The mutation profile of FHIT loss closely resembles the mutational signature 5 which suggests that loss of FHIT could be a marker for cancer development as well as a target for prevention of cancer initiation. Loss of FHIT expression occurs due to replication stress at the FHIT locus, at very low levels, which leads to genome -wide DNA instability in the FHIT deficient cells through the reduced expression of the TK1 gene which is needed for normal DNA replication. Genome instability and mutation accumulation in Fhit-deficient cells in tissue culture can be prevented through supplementation with low dose thymidine in the culture medium. It was tested to determine if low dose thymidine supplementation can prevent genome instability in vivo, reduce mutation accumulation and prevent development of cancerous lesions in FHIT-deficient versus sufficient mice, without causing imbalance of cellular dNTP pools for DNA synthesis, a possible deleterious side-effect. A preclinical mouse model using male and female mice ten weeks of age

and 50% each gender made up cohorts, eight mice each, which were FHIT +/+ or +/-. Half of each cohort (4 mice each) were treated with or without thymidine supplementation (1.8 g/kg thymidine). Mice receiving the diet without thymidine are pair-fed to allow ingestion of the same amount as thymidine supplemented mice. The mice were euthanized ten weeks after treatment and the tumor development was examined. Tumor burdens did not appear significantly different between +/+ and +/- mice. Histology must be done to further differentiate the tumor types. The research is innovative in using replacement of the scavenger thymidine pathway in vivo, a pathway lost as a direct result of FHIT loss, to counteract genome instability, a major downstream effect of lost FHIT genome caretaker and tumor suppressor function, and thus restore those functions to prevent tumor development.

INTRODUCTION

Findings leading to report of significant association of FHIT loss with signature 5 mutations: FHIT loss and signature 5 mutations occur in all types of cancer; occur early in the neoplastic process and are age-associated (3-8, 11); loss of FHIT causes genome-wide DNA instability (1, 2,11,12,13) and could, like loss/mutation of other caretaker genes, such as mismatch repair/BRCA1/2 genes, cause a specific mutational signature; the mutation profile of Fhit knockout mouse (ko) cells and tissues closely resembles mutational signature 5 (3, 5, 13) (see **Fig 1B**); the report of mutational consequences of smoking (14), comparing somatic mutations in smokers *vs* nonsmokers for smoking-associated cancers (**Fig 1,** for examples from ref 14); in **Fig 1B**, signature 5 shows mutations across all 96 mutation subtypes, with more T>C and C>T mutations, similar to the signature of Fhit -/- kidney and other tissues, also shown in **Fig 1B** (5,14**).** Signature 5 mutations were found in all cancer types studied in ref 14, including cancers of nonsmokers. Striking findings in **Fig 1** consistent with a role for FHIT/Fhit loss in production

of signature 5 mutations were: these alterations occur early in the preneoplastic process and appear as clonal alterations in a tumor, just as alterations within the fragile FHIT locus are clonal in cancers and cancer cell lines (3, 11, 14-16).

The discovery that Fhit loss is highly significantly associated with occurrence of mutational signature 5 in cancers suggested that loss of Fhit loss could be a marker for cancer development, as well as a target for prevention of cancer initiation (5).

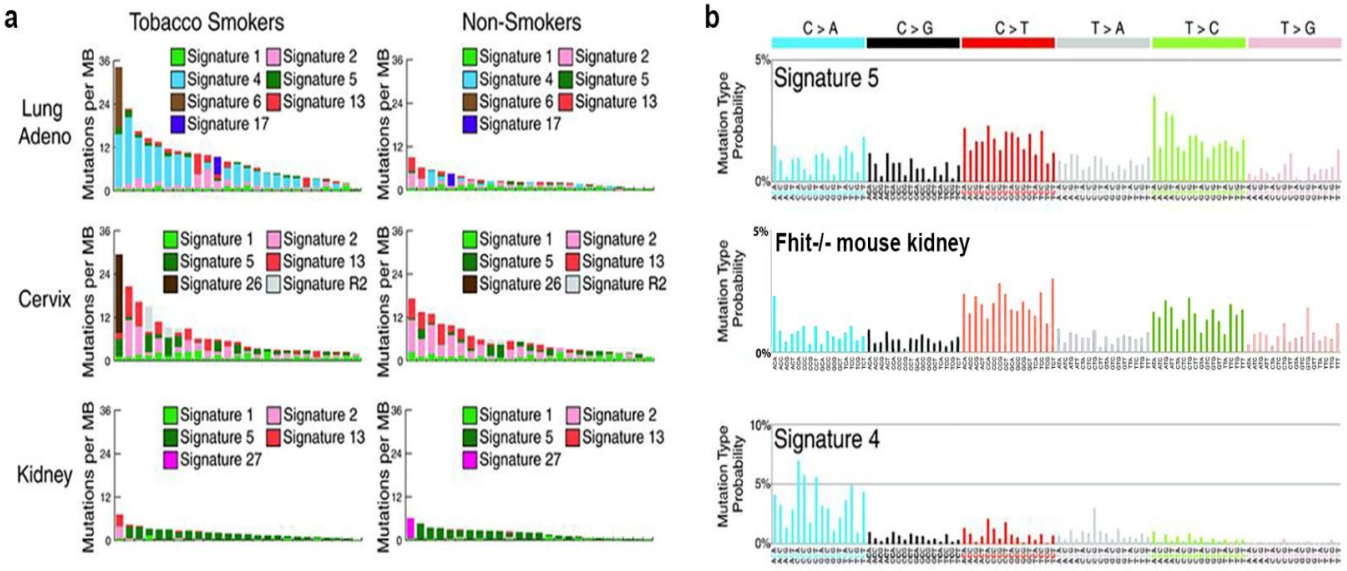

Fig 1. The mutational signatures in smoker's *vs* nonsmokers. An abbreviated copy of the "smoking signatures" from ref 14, to emphasize features of this signature that suggested FHIT loss as cause of mutational signature 5: A, the mutation spectra in 25 randomly selected cancer genomes (individual bars from smokers or nonsmokers of a given cancer type). Each bar is colored proportionately to the number of mutations/Mb of the specific mutational signatures found in the sample genome. Note signature 5 in kidney cancers; the FHIT gene was cloned due to a chromosome translocation within the gene in a hereditary kidney cancer family (16). B, patterns of mutational signatures 4 and 5 in tobacco smoker cancers as well as the mouse Fhit-/- signature in kidney tissue for comparison.

Loss of Fhit protein expression occurs due to replication stress at the FHIT locus, at very low levels in all individuals, likely in all normal tissues, and leads directly to genome-wide DNA

instability in the Fhit-deficient cells, through reduced expression of the TK1 gene, needed for normal DNA replication, followed by accumulation of mutations in those cells (1,2). Subsequent mutation in genes which promote selective growth or survival in those Fhit-deficient cells, allows clonal expansion that can lead to precancerous lesions and cancers, particularly on exposure to DNA damaging agents. We can stop this genome instability and mutation accumulation in Fhit-deficient cells in tissue culture through supplementation with low dose thymidine in the culture medium (1, 2). We will determine if low dose thymidine supplementation can prevent genome instability *in vivo*, reduce mutation accumulation and prevent development of cancerous lesions in Fhit-deficient *vs* sufficient mice, without causing imbalance of cellular dNTP pools for DNA synthesis, a possible deleterious side-effect. Significant differences in cancer development in association with thymidine supplementation, will provide preclinical support for planning of prevention trials in humans at high-risk for cancer development, possibly including BRCA1 mutation carriers, Barrett's esophagus patients at risk of progression to adenocarcinoma, or immunosuppressed transplant patients who may develop hundreds of precancerous skin or other lesions.

Since 2013 when the first 'mutational signatures' report (3) appeared, analysis of >10,000 sequenced cancers of 40 types has shown that the specific mutational signature 5, is clock-like (ie age associated), and occurs in all types of cancer (COSMIC website) (4). It has been reported that detection of this mutational signature in cancers is highly significantly associated with loss of expression of FHIT in these cancers (5). If it can be shown how to prevent Fhit-loss-induced genome instability, using mouse models carrying knocked-out Fhit or a conditionally expressed FhitTg in ko background, it will trail-blaze a prevention method that may be applicable to human preclinical trials, initially for high-risk cohorts and later possibly for other cancer types,

since Fhit loss has been observed in every cancer type examined, and leaves its telltale footprint, 'mutational signature 5'.

It is proposed that many of the 'unavoidable' mutations in cancer (9,10), such as the signature 5 mutations associated with FHIT loss (5), are due to the genome-wide DNA instability introduced through alterations at the FHIT/FRA3B fragile locus. It is hypothesize that, since Fhit-loss associated genome-wide accumulation of mutations is a direct result of Fhit-loss-induced down-modulation of Thymidine Kinase 1 (TK1) expression, needed for balanced intracellular thymidine pools for DNA synthesis, supplementation with low-dose thymidine could prevent development of genome instability and cancer development in mouse models exhibiting Fhit loss. Thus, if FHIT gene/Fhit protein expression loss or subsequent genome-wide DNA instability could be prevented, many 'unavoidable' mutations and resulting precancers will be avoided.

Aim: <u>Pharmacologic/Nutritional strategy</u>: Determine if thymidine supplementation suppresses Fhit loss-induced genome instability & tumor development in Fhit deficient mice.

Methods:

Protocol approved by the OSUMC IACUC. At the time of weaning (21 days of age), Aim 2 mice destined for the pharmacologic approach, are placed on standard diets with or without thymidine (1.8 g/kg thymidine, as described (31, 32), the dose used previously to evaluate the preventive effect of nucleoside–nucleotide supplementation on colonic mucosal damage in mice. Mice receiving the diet without thymidine are pair-fed to allow ingestion of the same amount as thymidine supplemented mice. Male and female mice 10 wks of age (20/genotype/procedure, 50% each gender); cohort sizes were determined by power analyses by Biostatistics co-I, Dr, Guy

Brock, as described below. *Smaller test cohorts of 8 mice per procedure with same timing and treatments, will be used for timed analysis of genome instability at 3- and 6-weeks post carcinogen (4 mice/cohort/*procedure). The mice for tumor endpoint assessment (20 mice/treatment cohort) were sacrificed at 10 weeks post-carcinogen and the tissues were harvested for histological, IHC and other analyses as described previously (17, 28). Esophageal and forestomach epithelia are prepared for DNA isolation by using a blade to remove submucosal and muscularis layers (33) and epithelial DNA prepared for gel mobility comparisons.

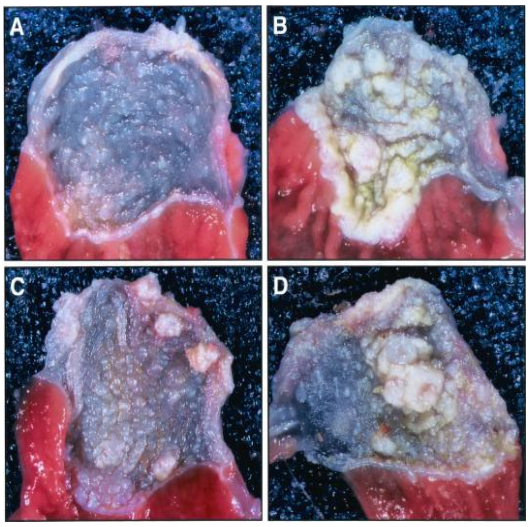

Fig 2. Gross anatomy of murine forestomach after NMBA treatment. Typical aspects of NMBA-induced pathology in forestomachs of *Fhit* +/+ mouse (*A*), *Fhit* +/− mouse (*B*), *Fhit* −/− mouse (*C*), and *Fhit* −/− mouse (*D*) are shown. (Magnification: ×5)

Tumorigenicity study. Mice of each genotype are produced in the in-house animal facility, treated with NMBA and analyzed as described previously (17). At autopsy, whole esophagi and forestomaches are removed and opened longitudinally for tumor count, inspection and fixation. The number of animals bearing tumors in the esophagus, forestomach, squamocolumnar junction with the glandular stomach are scored and differences in grossly observed lesion numbers are assessed using two-tailed Fisher's exact test. Tissues are fixed in buffered formalin and examined histologically after H&E staining for the presence and enumeration of hyperkeratosis, parakeratosis, dysplastic lesions, papillomas, adenomas, and carcinomas (as described in ref 17, 28, 33).

RESULTS AND DISSUCSSION

Upon dissection of the forestomaches, there was a visual difference between mice with the thymidine diets and the mice fed the normal diets treated with NMBA (**Fig 3**A). Both sets of mice

treated with NMBA showed thickening of the forestomaches but showed variability in number of tumors (**Fig** 3A). There was no difference in the types of tumors found between the two groups. It is suggested that a higher dose of thymidine may need to be administered in order to see statistical significance. **Fig 3B** showed a large subserosal lymph node aggregates present at the GE junction during histologic analysis. These seems to be larger and more present in FHIT +/- mice. Further studies must be done to show correlation.

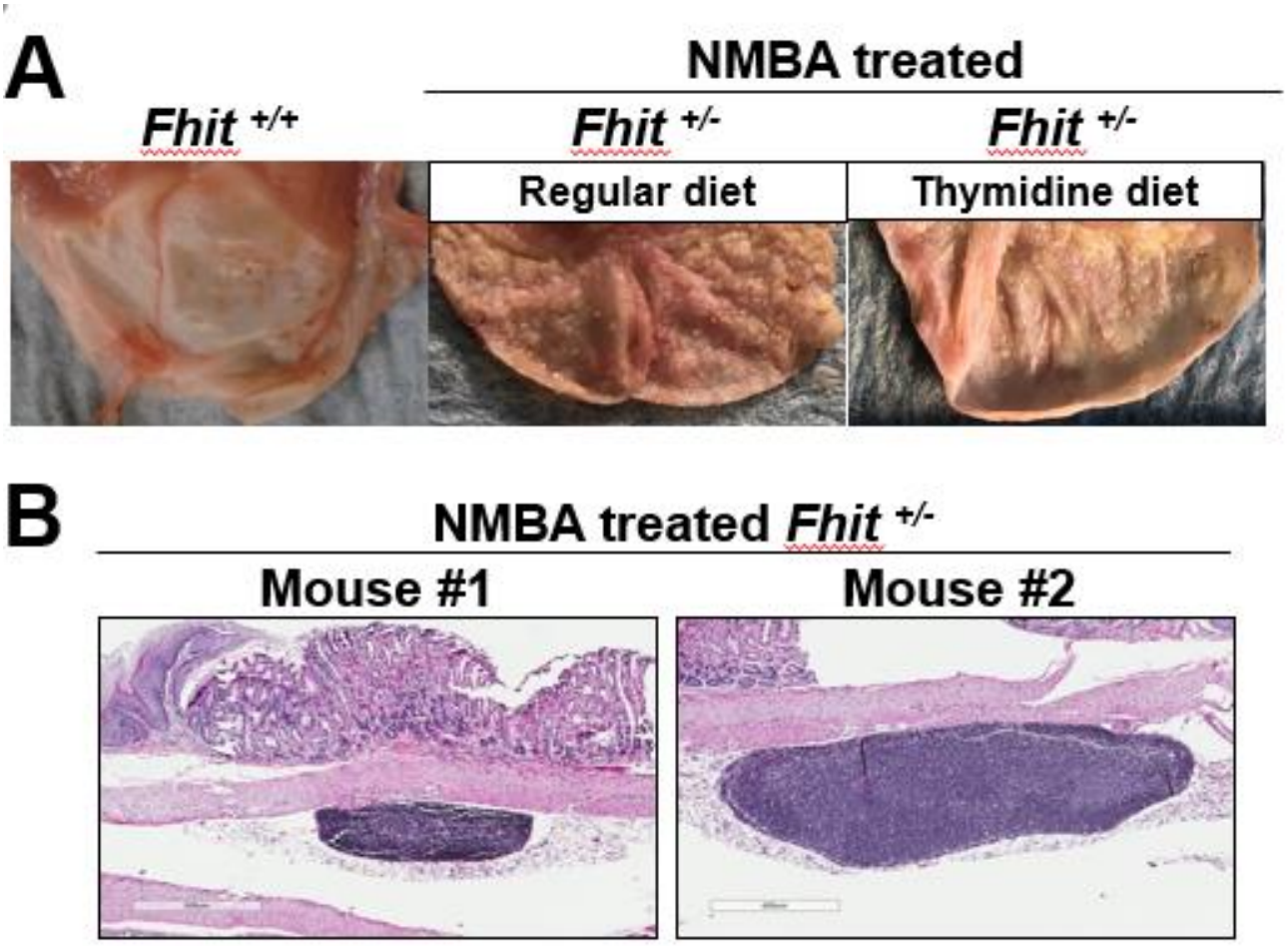

Fig 3: (A) Gross morphology photographs of forestomach of Fhit +/+ and NMBA treated Fhit +/- with and without thymidine diet. Some Fhit +/- animals NMBA treated with thymidine diet showed decreased tumor number and size. (B) Histologic analysis of mouse gastro-esophageal (GE) junction showed large subserosal lymph node aggregates present at the GE junction.

CONCLUSION

Higher dosage of Thymidine is needed to determine major difference between FHIT +/- and -/- mice in reducing NMBA-induced tumor growth. Some mice showed a reduction in tumor formation using a thymidine diet, however no major differences overall were shown. This suggests that the next step will be to increase the thymidine dose in order to see if thymidine can prevent genome instability.

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