Chapter 9: Paper 3

Presented in this chapter is the paper: "Point mutation instability (PIN) mutator phenotype as model for true back mutations seen in hereditary tyrosinemia type 1 – a hypothesis". This paper puts forward the hypothesis that the true back mutations as seen in HT1, is the result of a point mutation instability mutator phenotype. The paper was published in *Journal of Inherited Metabolic Disease*.

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HYPOTHESIS

Point mutation instability (PIN) mutator phenotype as model for true back mutations seen in hereditary tyrosinemia type 1 – a hypothesis

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Abstract Hereditary tyrosinemia type 1 (HT1) is an autosomal recessive disorder affecting fumarylacetoacetate hydrolase (FAH), the last enzyme in the tyrosine catabolism pathway. The liver mosaicism observed in HT1 patients is due to the reversion to the wild type of one allele of the original point mutation in *fah*. It is generally accepted that these reversions are true back mutations; however, the mechanism is still unresolved. Previous reports excluded intragenic recombination, mitotic recombination, or homologous recombination with a pseudogene as possible mechanisms of mutation reversion in HT1. Sequence analysis did not reveal DNA motifs, tandem repeats or other sequence peculiarities that may be involved in mutation reversion. We propose the hypothesis that a point mutation instability mutator (PIN) phenotype brought about by the sustained stress environment created by the accumulating metabolites in the cell is the driver of the true back mutations in HT1. The metabolites accumulating in HT1 create a sustained stress environment by activating the extracellular signal-regulated kinase (ERK) and AKT survival pathways, inducing aberrant mitosis and development of death resistant cells, depleting glutathione, and impairing DNA ligase IV and possibly DNA polymerases δ and ε. This continual production of proliferative and stress-related survival signals in the cellular environment coupled with the mutagenicity of FAA, may instigate a mutator phenotype and could end in tumorigenesis and/or mutation reversion. The establishment of a PIN-mutator phenotype therefore not only seems to be a possible mechanism underlying the true back mutations, but also contributes to explaining the clinical heterogeneity seen in hereditary tyrosinemia type 1.

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Background

Hereditary tyrosinemia type 1 (HT1, OMIM 276700), an autosomal recessive disorder affecting fumarylacetoacetate hydrolase (FAH, E.C. 3.1.7.2), is characterized by accumulating metabolites such as fumarylacetoacetate (FAA), maleylacetoacetate (MAA), succinylacetone (SA), p-hydroxyphenylpyruvic acid (pHPPA), p-hydroxyphenylacetic acid (pHPAA) and p-hydroxyphenyllactic acid (pHPLA) (Mitchell et al. 2001).

Most HT1 patients present with symptoms within the first few months of life but some only after 6 months (Mitchell et al. 2001). A high incidence of hepatocellular carcinoma (HCC) is found in HT1 (Mitchell et al. 2001), but the risk is reduced by early treatment with NTBC (Fernandes et al. 2006). In the majority (~83-88%) of HT1 patient livers, FAH-immunopositive nodules are found, comprised of cells that apparently function normally (Kvittingen et al. 1994, Youssoufian and Pyeritz 2002, Demers et al. 2003), thereby creating a liver mosaic. This mosaicism is due to the reversion to the wild type of one allele of the original point mutation in *fah* (RefSeq NM_000137.2), which sufficiently re-establishes a normal metabolic milieu giving such cells a growth advantage over cells with non-corrected mutations (Kvittingen et al. 1994). Although reversion was observed for only some of the HT1 mutations, i.e. IVS12+5 g>a, G337S, Q64H and Q279R (Kvittingen et al. 1994, Demers et al. 2003), reversion of

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other HT1 mutations cannot be excluded. Except for one Norwegian patient, where a triple mosaic was observed (Bliksrud et al. 2005), only one type of reversion is reported in each individual.

The extent of the mutation reversion varies between 0.1-85%, and a correlation appears to exist between the extent of the particular reversion, the age of the patient and the severity of the disease, which may lead one to speculate that the number of reversions may increase with the duration of the disease (Demers et al. 2003).

Point mutation reversion mosaicism is also seen in other inherited disorders such as Adenosine Deaminase Deficient Severe Combined Immunodeficiency (ADA-SCID, OMIM 102700), Bloom Syndrome (OMIM 210900), Epidermolysis Bullosa (OMIM 113811 and 1408066) and Fanconi Anaemia (OMIM 227650) (Youssoufian and Pyeritz 2002, Hirschhorn 2003). Proposed mechanisms to explain these reversions include true back mutations, homologous recombination, substitutions in the mutant codon, second-site repressor mutations, transpositions or changes in the activity of mobile elements in the genome, epigenetic alterations of DNA and chromosomal abnormalities (Kvittingen et al. 1994, Youssoufian and Pyeritz 2002). This diversity of mechanisms suggests that there is not a single mode of action to collectively explain all these reversions.

Although it is generally accepted that the HT1 reversions are true back mutations of one allele to the wild type sequence (Kvittingen et al. 1994, Bliksrud et al. 2005, Hirschhorn 2003), the underlying mechanism is still unresolved.

Previous reports suggested that the stress environment created by the accumulating metabolites could contribute to the development of HCC and contribute to the high rate of mutation reversion in HT1 (Demers et al. 2003, Jorquera and Tanguay 1997).

We want to elaborate on these suggestions and hypothesise that a point mutation instability mutator phenotype (PIN phenotype), brought about by a sustained intracellular stress environment induced by the accumulating metabolites, is the driver of the true back mutations in HT1.

Presentation of the hypothesis

Establishing a sustained stress environment

In HT1 the accumulating metabolites creates and maintains an intracellular stress environment in all the cells (Bergeron et al. 2006).

The accumulating FAA is mutagenic, which is potentiated by its glutathione depleting properties (Jorquera and Tanguay 1997). Since depletion of GSH causes a pronounced retardation in DNA repair and an imbalance in GSH levels are implicated in carcinogenesis (Townsend et al. 2003), tight regulation of GSH levels is important. FAA, also activates signals such as ERK and the AKT survival pathway, induces apoptosis and aberrant mitosis, endoplasmic reticulum stress, and tumour hypoxia (Bergeron et al. 2006, Jorquera and Tanguay 1999, Jorquera and Tanguay 2001, Orejuela et al. 2008, Edwards et al. 1991). In itself, hypoxia causes genomic instability by down-regulating at least five DNA repair genes involved in mismatch repair and homologous recombination double-strand break repair (Galhardo et al. 2007).

The HT1 pathognomonic metabolite, SA, may have an effect on DNA polymerases ε and δ. This is derived from the report that SA inhibits DNA ligase activity (Prieto-Alamo and Laval 1998) by forming stable adducts with lysine (Manabe et al. 1985). This reactivity of SA may therefore have an effect on the activity of the exonuclease domains of DNA polymerases δ and ε, since they both contain a highly conserved lysine amino acid (de Vega et al. 1997). Hampered exonuclease activity plays an important role in cancer development (Reha-Krantz 2010). A proof-reading relationship exists between DNA polymerase ε and δ (Morrison and Sugino 1994) since an exonuclease deficiency in either DNA polymerase δ or ε results in a synergistic increase in mutation rates (Morrison and Sugino 1994). It was furthermore shown that mutations of the Lys143 of φ29 DNA polymerase (a B family DNA polymerase, like δ and ε) cause reduced 3'-5' exonuclease activity and result in a mutator polymerase (de Vega et al. 1997). If either polymerase is affected, a scenario resembling a single-allele mutation might unfold. The change(s) that result from the affected DNA polymerase may confer to the daughter cell a higher susceptibility to the establishment of a PIN mutator phenotype or even a mutation reversion.

We have furthermore observed the impairment of DNA repair in liver cells through decreased functionality of the proteins involved in the initial steps of base- and nucleotide excision repair pathways by p-hydroxyphenylpyruvic acid (van Dyk et al. 2010).

From the above it is clear that the accumulating metabolites not only place an increased burden on genome stability, through their mutagenicity, but also negatively affect the DNA repair mechanisms responsible for the repair of the DNA lesions. As HT1 cells may also be resistant to cell death, this doubly negative effect may lead to an increase in mutations.

Effect of a point mutation mutator (PIN) phenotype

A mutator phenotype may be activated in cells experiencing a continual production of proliferative and stress-related survival signals. A mutator phenotype was originally

defined as an error-prone progression of the cell-cycle involving error-prone polymerases and mitosis, but now includes all sources of heritable changes comprising genes involved in microsatellite, chromosomal and checkpoint instability and maintenance of the epigenome (Loeb et al. 2008, Beckman 2010).

We propose that the metabolite induced sustained stress environment impairs the function of the proteins guarding the fidelity of the genetic material, and in doing so establish a point mutation instability (PIN) mutator phenotype (Bielas et al. 2006).

To further elaborate on this proposed mechanism, we would like to expand on a proposal by Kvittingen (Kvittingen et al. 1994) and base our argument on a sequence of events suggested by Bignold (Bignold 2007) for establishing genomically stable tumors. Since HCC develops and progresses through the accumulation of several genetic changes (Saffroy et al. 2004) the efficiency advantage of a mutator pathway is large (Beckman 2010). Efficiency is defined by Beckman as "the expected number of malignant lineages which could be generated by the particular mechanism on or before the time of life (in cell generations) when a particular malignancy is typically observed" (Beckman 2010). The mutator phenotype can therefore cause a mutation that either contributes to the development of the HCC frequently seen in HT1 or occurs at such a location as to cause a mutation reversion (Bielas et al. 2006, Bignold 2007). As this event is random in nature (Triampo et al. 2007), the possibility exists that by chance a HT1 mutation is the target whereby the wild type sequence is re-established in one allele (Fig. 1). The triple mosaicism observed in the Norwegian patient (Bliksrud et al. 2005) furthermore suggest a cooperative lineage expansion model, since a cooperative lineage expansion model leads to mosaic populations of cells with varying numbers of cells and lineages (Beckman 2010).

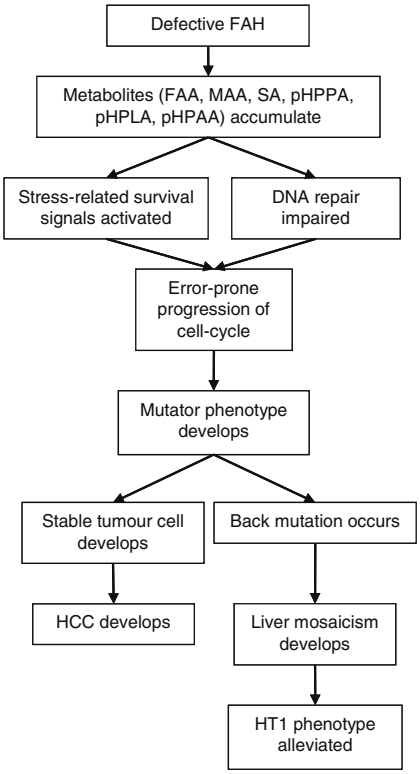

Fig. 1 Overview of the development and outcomes of a PIN mutator phenotype in HT1. The accumulating intermediates contribute to the creation of a stress-environment. And sustaining this stress-environment causes an error-prone progression of the cell-cycle. A PIN mutator phenotype ensues, which could result either in a true back mutation arising or the development of a stable tumour cell. If a true back mutation arises, the stress-environment in the cell is lessened and the cell gains a growth advantage, leading to the formation of a liver mosaicism

Factors influencing the rate at which a PIN mutator phenotype is established

A number of factors can influence the rate at which mutation reversion occur. Firstly, the configuration in which the bases occur can influence their incorporation during DNA replication. Spontaneous transitions and transversions can occur when non-Watson-Crick base-pairs are formed, which are then missed during proofreading as they mimic Watson-Crick geometry (Triampo et al. 2007). However, this non-Watson-Crick base-pairing is very rare (Triampo et al. 2007). Secondly, the mutation rate may be influenced by the sequence context in which the point mutation arises (Walser et al. 2008). The most commonly known of these sequence contexts in mammals are the CpG dinucleotides, where the methylated cytosine in the CpG site has a high mutation rate (Ellegren et al. 2003). Although none of the reported HT1 reverting mutations occurs in a CpG site, their mutation rate may be contingent on the local CpG content, the chromosomal location of *fah*, and the GC content of the sequence (Walser et al. 2008, Ellegren et al. 2003). Most mutations that occur in a non-CpG site, however, are believed to occur due to DNA replication errors (Walser et al. 2008, Seo et al. 2000). The mutation frequency might also be different between leading- and lagging-strand synthesis due to differences in the DNA polymerase and/or its auxiliary factors (Seo et al. 2000). Polymerase ε, the leading strand synthesis polymerase, has an error-rate of $\leq 0.2 \times 10^{-5}$ substitutions and 0.05×10^{-5} insertions or deletions, and polymerase δ, the lagging strand synthesis polymerase, has an error rate of $<1.3 \times 10^{-5}$ substitutions and 1.3×10^{-5} insertions or deletions (Hoffmann and Cazaux 2010). If SA affects the activities of DNA polymerase δ and ε, an increase in the error-rate of these polymerases may occur. Under normal circumstances, the errors that escape the proofreading of these DNA polymerase, are repaired by the mismatch repair

pathway (Preston et al. 2010). However, currently there is no data available on the effect on the mismatch repair pathway in HT1. In addition, base excision repair is responsible for the repair of the approximately 2 x 10^4 spontaneous DNA lesions that arise per mammalian cell per day (Preston et al. 2010). In HT1, the mutation rate may be higher, due to the high mutagenicity of FAA (Jorquera and Tanguay 1997). Therefore, if the BER capacity of the cell is affected, like in HT1 (van Dyk et al. 2010), these lesions are repaired at a reduced rate, creating an accelerated rate development of a PIN mutator phenotype.

The PIN-mutator phenotype might also be intensified by epigenetic events, such as DNA methylation which alters the sensitivity of DNA to mutation, thereby rendering genes more susceptible to damage by toxic compounds (Dixon and Kopras 2004). Hypomethylation of DNA is causally linked to tumorigenesis, possibly through chromosomal instability (Gaudet et al. 2003). In line with the above, we have observed deranged global DNA methylation in HT1 patients (Wentzel et al. 2010).

Glutathione depletion also causes global epigenetic alterations. Epigenetic alterations have long been linked with the development of cancer (Esteller 2008). These molecular events undoubtedly play a crucial role in the proposed model to explain mutation reversion in HT1.

PIN mutator phenotype as model for true back mutation

In conclusion, we therefore propose that the metabolites accumulating in HT1 create a sustained stress environment that impairs the DNA repair machinery that in turn results in the establishing of a PIN mutator phenotype. From this PIN mutator phenotype, a mutation may arise that either contributes to the establishment of a stable tumour cell, or if one of the HT1 mutations was the target, the wild type sequence is restored, thereby restoring enzyme function. Several factors may influence the rate at which the reversion occurs and the existence of strong and mild mutator phenotypes may also contribute to the eventual extent of the HT1 phenotype (Bignold 2006).

Evaluation of the hypothesis

To evaluate the proposed hypothesis we investigated the possible involvement of mutation hotspots or the sequence context dependence of the reversion (Jackson and Loeb 2001). A recent study suggested that the probability of a mutation at a specific site might be influenced by the polymorphic sites in its vicinity (Tian et al. 2008). We therefore compared the DNA sequences surrounding the point of reversion in HT1 and the other mentioned diseases with reverting mutations to unveil peculiarities in the sequence context that may shed light on the mechanism of reversion.

In the 100 bp DNA sequences surrounding the reported reverting mutations, apart from the cap-signal (NCANNNNN) which initiates transcription, no apparent recurring motifs were observed. No tandem repeats or mutation hotspots and no obvious similarities were observed in the DNA flanking sequences amongst the different mutations investigated. DNA motifs, i.e. structural or regulatory motifs, tandem repeats or sequence similarity that could lead to mutation vulnerability or genetic instability can, therefore, not form the basis of a mechanism of reversion of the inherited mutations. No clustering of polymorphisms around the reverting mutations, that could have indicated a higher probability of mutation reversion (Tian et al. 2008), was observed.

We did not find any other similarities on metabolic, genomic or tissue level amongst the inherited disorders displaying mosaicism. It seems safe to conclude that the mechanism of the mutation reversions occurring in these diseases is rather disease specific and that no apparent DNA sequence feature(s) is conducive to mutation reversions. Since some of the patients are homozygous for the mutations, reversion to wild-type via intragenic recombination or mitotic recombination can be excluded, as can the reversion of mutations via homologous recombination of *fah* with a pseudogene, since there is no known pseudogene for *fah* (Hirschhorn 2003).

Given the above mentioned, the establishment of the PIN mutator phenotype via impairment of DNA repair, therefore seems a likely mechanism underlying the reversion of the mutation to wild-type as seen in hereditary tyrosinemia type 1.

Supporting this hypothesis is the observations of impaired DNA repair capacity in hereditary tyrosinemia type 1 (Prieto-Alamo and Laval 1998, van Dyk et al. 2010). In addition, it is reported that point mutation instability are seen in parallel with microsatellite and chromosomal instability in human cancers (Bielas et al. 2006). Chromosomal instability were reported for HT1 (Jorquera and Tanguay 2001, Gilbert-Barness et al. 1990, Zerbini et al. 1992).

Implications of the hypothesis

There is currently is no apparent correlation between the genotype and the phenotype of HT1, but the establishment of a PIN-mutator phenotype may unveil aspects of the clinical heterogeneity observed (Mitchell et al. 2001).

Although, reversion of mutations and the creation of a mosaic are not an instant cure for otherwise harmful disorders (Youssoufian and Pyeritz 2002), our hypothesis

would aid researchers in determining not only the mechanism by which the true back mutations arise in HT1, but also the mechanism by which the HCC develops. This knowledge may then eventually lead to treatment therapies that delay or abolish the onset of hepatocellular carcinoma, such as suggested by Fox and Loeb (Fox and Loeb 2010).

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