The molecular basis of the genetic mosaicism in hereditary tyrosinemia (HT1)

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Abstract

Hereditary tyrosinemia type 1 (HT1) is an autosomal recessive disorder of the tyrosine degradation pathway. The defective fumarylacetoacetate hydrolase enzyme causes the accumulation of upstream metabolites such as fumarylacetoacetate (FAA), maleylacetoacetate (MAA), succinylacetone (SA) and *p*-hydroxyphenylpyruvic acid (pHPPA). *In vitro* and *in vivo* studies showed that the accumulation of these metabolites are detrimental to cell homeostasis, by inducing cell cycle arrest, apoptosis, and endoplasmic reticulum stress, depleting GSH, inhibiting DNA ligase, causing chromosomal instability, etc. For *in vivo* studies different models of HT1 were developed. Most notably was the *fah* deficient mouse, whose neonatally lethal phenotype is rescued by the administration of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC). Although, this model most closely resembles the human phenotype with elevated tyrosine levels and the development of hepatocellular carcinoma (HCC), the model is not human genome based.

Both the *in vitro* and *in vivo* studies suggested that DNA repair is affected in HT1. However, it is not yet clear which DNA repair mechanisms are affected and if only protein functionality is affected, or if expression of DNA repair proteins are also affected.

Characteristic of HT1 is the high prevalence of HCC and the presence of liver mosaicism. The liver mosaicism observed in HT1 patients are the result of reversion of the inherited mutation to wild-type. The general consensus is that the reversion is the result of a true back mutation. However, the mechanism underlying the back mutation is still unresolved.

It was suggested that cancer develops either through a chromosomal instability mutator phenotype, a microsatellite instability mutator phenotype, or a point mutation instability mutator phenotype. In HT1 only chromosomal instability was reported.

The aims of this study were to contribute to the understanding of the molecular basis of the genetic mosaicism in hereditary tyrosinemia type 1. More specifically, determine whether base- and nucleotide DNA repair mechanisms are affected and to what extent, and to determine if microsatellite instability is found in HT1. To achieve these aims, a parallel approach was followed: i.e. to develop a HT1 hepatic cell model and to use HT1 related models and HT1 patient material. To assess the molecular basis of the genetic mosaicism in HT1, the comet assay, gene expression assays, microsatellite instability assays, high resolution melting and dideoxy sequencing techniques were employed.

Results from the comet assay showed that the HT1 accumulating metabolites, SA and pHPPA, decreased the capacity of cells for base- and nucleotide excision repair. Gene expression assays showed that short term exposure to SA and/or pHPPA do not affect expression of *hOGG1* or *ERCC1*. The expression of these genes were, however, low in HT1 patient samples. Microsatellite instability assays showed allelic imbalance on chromosome 7 of the mouse genome, and microsatellite instability in the lymphocytes of HT1 patients. Although high resolution melt and sequencing results did not reveal any *de novo* mutations in *fah* or *hprt1*, the appearance of *de novo* mutations on other parts of the genome can not be ruled out.

To conclude, results presented in this thesis, for the first time show that in HT1 the initiating proteins of the base- and nucleotide repair mechanisms are affected, the gene expression of DNA repair proteins are low, and microsatellite instability is found in HT1. By contributing to the elucidation of the mechanism underlying the development of HT1-associated HCC, and providing evidence for the development of a mutator phenotype, the results presented in this thesis contributes to the understanding of the molecular mechanisms underlying the genetic mosaicism in HT1. In addition to these contributions, a hypothesis is posited, which suggests that a point mutation instability (PIN) mutator phenotype is the mechanism underlying the mutation reversions seen in HT1.

Samevatting

Die onderwerp van hierdie studie is: “Die molekulêre agtergrond van die genetiese mosaïek in oorerflike tirosinemie (HT1)”. Oorerflike tirosinemie tipe 1 (HT1) is 'n outosomale resessiewe versteuring van die tirosien-katabolisme. Die defektiewe fumarielasetoasetaat-hidrolase ensiem veroorsaak dat intermediêre metaboliete soos fumarylasetoasetaat, maleïelasetoasetaat, suksinielasetoon (SA) en *p*-hidroksiefenielpiruvaat (pHPPA) ophoop. Beide *in vitro* en *in vivo* studies het getoon dat die opeenhoping van hierdie metaboliete nadelig is vir sel-homeostase deur onder andere staking van die selsiklus, apoptose, en endoplasmiese retikulum stres te induseer, glutatioonvlakke uit te put, DNS-ligase te inhibeer en chromosomale-onstabiliteit te veroorsaak. Vir *in vivo* studies is verskillende HT1-modelle ontwikkel, waarvan die belangrikste die *fah*-gebrekkige muis is, en die neonatale fenotipe van hierdie model word grootliks beskerm deur behandeling met NTBC. Alhoewel hierdie model baie ooreenstem met die menslike fenotipe, met verhoogde tirosien-vlakke en die ontwikkeling van HCC, is die model nie op die menslike genoom gebaseer nie.

Beide *in vitro* en *in vivo* studies dui daarop dat DNS-herstel benadeel word in HT1, maar dit is egter nog nie duidelik watter DNS-herstelmeganismes geraak word nie, en of slegs proteïenfunksie, of uitdrukking van DNS-herstelproteïene ook geraak word nie.

Kenmerkend van HT1 is die hoë voorkoms van hepatosellulêre karsinoom (HCC) en die teenwoordigheid van ‘n lewermosaïek. Die lewermosaïek wat in HT1 pasiënte waargeneem word, is die gevolg van die omkeer van die oorgeërfde mutasie tot die wilde-tipe. Die algemene konsensus is dat hierdie omkeer die resultaat is van 'n ware terugmutasie. Die meganisme onderliggend hieraan is egter nog onbekend.

Die algemene oortuiging is dat kanker kan ontwikkel óf deur ‘n chromosomale-onstabiliteit gegenereerde mutasie-fenotipe óf 'n mikrosatelliet-onstabiliteit genereerde mutasie-fenotipe. In HT1 is daar al chromosomale-onstabiliteit waargeneem, maar nie mikrosatelliet-onstabiliteit nie.

Die doel van hierdie studie was om by te dra tot die ontrafeling van die molekulêre basis van die genetiese mosaïek wat voorkom in tirosinemie tipe 1. Meer spesifiek, om vas te stel of basis- en nukleotied DNS-herstelmeganismes beïnvloed word en tot in watter mate, en om te bepaal of mikrosatelliet-onstabiliteit in HT1 waargeneem word. Om hierdie doelwitte te bereik is 'n parallelle benadering gevolg. Aan die een kant is 'n HT1 hepatiese sel model ontwikkel deur RNS-tussenkoms tegnologie, en aan die ander kant is van HT1-verwante modelle en HT1-

pasiëntmateriaal gebruik gemaak. Om te molekulêre basis van die genetiese mosaïek in HT1 te bepaal is die volgende analises uitgevoer: die komeet analise, geenuitdrukking analise, mikrosatelliet-onstabiliteit analise, hoë resolusie smelt, en volgordebepalings.

Resultate van die komeet analise het getoon dat die HT1-akkumulerende metaboliete, SA en pHPPA, die vermoë van selle vir basis- en nukleotied DNS-herstel verlaag. Geenuitdrukkings analises het getoon dat die korttermyn blootstelling aan SA en/of pHPPA nie die uitdrukking van *hOGG1* of *ERCC1* beïnvloed nie. Die uitdrukking van hierdie gene is egter aansienlik verlaag in HT1-pasiëntmateriaal. Mikrosatelliet-onstabiliteit analises het getoon dat alleliese-wanbalans in chromosoom 7 van die muis genoom, en mikrosatelliet-onstabiliteit in die limfosiete van HT1-pasiënte voorkom. Alhoewel in die hoë resolusie smelt en volgordebepalings resultate *de novo* mutasies in *fah* of *hprt1* nie waargeneem is nie, kan die voorkoms van *de novo* mutasies in ander dele van die genoom nie uitgesluit word nie.

Die resultate wat in hierdie proefskrif vervat is, wys vir die eerste keer dat in HT1, basis- en nukleotied DNS-herstelmeganismes geraak word, die geen uitdrukking van DNS-herstelproteïene verlaag is, en dat mikrosatelliet-onstabiliteit in HT1 waargeneem word. Deur by te dra tot die toeligting van die meganismes onderliggend aan die ontwikkeling van HT1-verwante HCC, en die verskaffing van bewyse vir die ontwikkeling van 'n mutasiegenererende fenotipe, dra die resultate by tot die begrip van die molekulêre meganismes onderliggend aan die genetiese mosaïek gesien eie aan HT1. Bo en behalwe vir hierdie bydraes, is 'n hipotese gemaak dat ‘n puntmutasie-onstabiliteit (PIN) mutasiegenererende fenotipe die meganisme onderliggend is aan die mutasie omkerings wat in HT1 mag plaasvind.

Keywords

Hereditary tyrosinemia type1; Mosaicism; Hepatocellular carcinoma; DNA repair; Genome instability; Mutator phenotype

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List of symbols

℃:	Degrees Celsius
µl:	Microlitre
µM:	Micromolar
α:	Alpha
β:	Beta
Δ or δ:	Delta
ε:	Epsilon
%:	Percentage
φ:	Phi

List of abbreviations

A:

A:	Adenine
ANON:	Anonymous
ANOVA:	Analysis of variance
AP:	Abasic site
APC:	Adenomatous polyposis coli
ATCC:	American tissue culture company
ATP:	Adenosine triphosphate

B:

BER:	Base excision repair
BiP:	Binding immunoglobulin protein
bp:	Basepair
BRCA1:	Breast cancer 1
BSA:	Bovine serum albumin

C:

C:	Cytosine
CAF:	Central Analytical Facilty
cDNA:	Complementary DNA
CHOP:	CEBP homologous protein
CIN:	Chromosomal instability
cM:	Centi-Morgan
COLD-PCR:	Co-amplification at lower denaturation temperature-PCR
CSA:	Cockayne syndrome group A
CSB:	Cockayne syndrome group B
Cu:	Copper

D:

DDB:	DNA damage binding protein
ddH_2O:	Double distilled water
DMEM:	Dulbecco's modified eagles medium
DMSO:	Dimethylsulfoxide
DNA:	Deoxyribonucleic acid
DNMT1:	DNA methyltransferase 1
dNTP:	Deoxyribonucleotide triphosphate
DOPA:	Dihydroxyphenylalanine

DOVA: 4,5-Dioxovaleric acid
Dox: Doxycycline
DSB's: Double strand breaks
DTT: Dithiothreitol

E:
EDTA: Ethylenediamine tetraacetic acid
EGTA: Ethylene glycol-bis(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
eIF2α: Eukaryotic translation factor 2 alpha
ENU: *N*-ethyl-*N*-nitrosourea
ERCC1: Excision repair cross-complementing rodent repair deficiency, complementation group 1
ERK: Extra cellular signal-regulated protein kinase
Et al: Et Alii/Alia (Latin: And others)
EtBr: Ethidium Bromide
EtOH: Ethanol
EXO1: Exonuclease 1

F:
FA: Fumarylacetone
FAA: Fumarylacetoacetate
FAH: Fumarylacetoacetate hydrolase
FBS: Fetal bovine serum
Fe: Iron
FPG: Formamido-pyrimidine glycosylase
Fwd: Forward

G:
g: Gram
g: Gravitational force
G: Guanine
Gas: Growth arrest specific 2
GGR: Global genomic repair
GRP17: Gadd-related protein 17 kDa
GSH: Glutathione
GSTZ1-1: Glutathion transferase zeta

H:
h: Hours
H_2O: Water
H_2O_2: Hydrogen peroxide
HCC: Hepatocellular carcinoma

HEPES:	N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid
HGD:	homogentisate-1,2-dioxygenase
HMPA:	High melting point agarose
HPD:	4-hydroxyphenylpyruvate dioxygenase
HPRT1:	Hypoxanthine phosphoribosyltransferase 1
HR:	Homologous repair
HRM:	High resolution melt
HT1:	Hereditary tyrosinemia 1

I:

i.e.:	id est (that is)

K:

KCl:	Potassiumchloride
$(KH_2P)O_4$:	Potassiumdihydrogen orthophosphate

L:

LMPA:	Low melting point agarose

M:

3-meA:	3-Methyladenine
7-meG:	7-Methylguanine
M:	Molar
MA:	Maleylacetone
MAA:	Maleylacetoacetate
MEM:	Minimum essential medium Eagle
mg:	Milligram
min:	Minutes
miRNA:	Micro RNA
ml:	Millilitre
MLH:	MutL homolog
mM:	Millimolar
MMLV:	Moloney Murine Leukemia Virus Reverse Transcriptase
MMR:	Mismatch repair
mRNA:	Messenger RNA
MSH:	MutS homolog
MSI:	Microsatellite instability
MutLα:	MLH1•PMS2 heterodimer
MutSα:	MSH2•MSH6 heterodimer

N:

N.A.	Not available
NaCl:	Sodium chloride
$NaHCO_3$:	Sodium bicarbonate
NaOH:	Sodium hydroxide
NCI:	National Cancer Institute
N.D.:	Not done
NER:	Nucleotide excision repair
ng:	Nanogram
NHEJ:	Non-homologous end joining
NTBC :	2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione

O:

OH:	Hydroxy radical
8-oxoG:	8-Oxoguanine

P:

PAGE:	Polyacrylamide gel electrophoresis
PBS:	Phosphate buffered saline
PCR:	Polymerase chain reaction
pH:	Potential of Hydrogen
pHPAA:	p-Hydroxyphenylacetic acid
pHPLA:	p-Hydroxyphenyllactic acid
pHPPA :	p-Hydroxyphenylpyruvic acid
PIN:	Point mutation instability
PKU:	Phenylketonuria
PMS2:	postmeiotic segregation increased 2

R:

Ref:	Reference
Rev:	Reverse
RFLP:	Restriction fragment length polymorphism
RNA:	Ribonucleic acid
RNAi:	RNA interference
RNAPII:	RNA polymerase II
ROS:	Reactive oxygen species
RPA:	Replication protein
RPM:	Revolutions per minute
RQ:	Relative quantification
rRNA:	Ribosomal RNA
RT-PCR:	Reverse transcription polymerase chain reaction

S:

SA:	Succinylacetone
SAA:	Succinylacetoacetic acid
SCGE:	Single cell gel electrophoresis
sec:	Seconds
SFM:	Serum free medium
shRNA:	Short hairpin RNA
siRNA:	Small interfering RNA
SOC:	Super optimal broth with catabolite repression
SSB:	Single strand break

T:

T:	Thymine
T_c:	Critical melting temperature
TCR:	Transcription coupled repair
Tet:	Tetracycline
TFIIH:	Transcription factor II H
T_m:	Melting temperature
Tris-HCl:	2-Amino-2-(hydroxymethyl)-1,3-propandiol-hydrochloride
tTS:	Tetracycline-controlled transcriptional suppressor

U:

U:	Uracil
U:	Units
UV:	Ultra violet

X:

XPA:	Xeroderma pigmentosum, complementation group A
XPC:	Xeroderma pigmentosum, complementation group C
XPF:	Xeroderma pigmentosum, complementation group F
XPG:	Xeroderma pigmentosum, complementation group G
XRCC1:	X-ray repair complementing defective repair in Chinese hamster cells 1

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