Concordant Inactivated X-Chromosome Results from Early Monoclonal Expansions of Neural Crest Precursors in MEN-2A

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Background: The histologic definition of precursor lesions in MEN is controversial for both C-Cell Hyperplasia (CCH) and Adrenal Medullary Hyperplasia (AMH). They are multifocal and the clonality pattern of each focus remains unknown.

Methods: We found 10 females with bilateral CCH and another 11 with AMH in a MEN-2A kindred known to carry *RET* point mutation in codon 634. DNA was extracted from microdissected samples of CCH from each thyroid lobe and from 34 AMH nodules (2 nodules in 3 patients, 3 nodules in 4, and 4 nodules in 4). The samples were then used to study the methylation pattern of X-chromosome inactivation (HUMARA test using Hha-I digested and undigested samples). Appropriate and multiple tissue controls were run in every case. The inactivation pattern of X-chromosome of each sample was compared for any given patient, including only informative cases (2 alleles in both undigested and digested control samples) in the final analysis.

Results: Nine CCH patients were informative, 8 of them revealing monoclonal pattern with the same androgen receptor allele preferentially methylated in both lobes. Twenty-seven of 30 AMH nodules from 9 informative patients also revealed monoclonal unbalanced methylation of androgen receptor alleles and the same X-chromosome inactivated in nodules from a given patient. The remaining 2 CCH foci (1 patient) and 3 AMH nodules (2 patients) revealed balanced methylation of androgen receptor alleles. Under the hypothesis of independent allele methylation in different lesions from the same patient, the combined probability of randomly finding the observed monoclonal results was less than 0.0001 for CCH and less than 0.000000001 for AMH.

Conclusions: MEN-2A-related CCH and nodular AMH are mainly monoclonal, and show the same X-chromosome inactivated in both thyroid lobes and in adrenal nodules from a given patient. This suggests a multifocal origin for both conditions related to early clonal expansions of neural crest precursors and may represent a paradigm for other germline mutations during embryogenesis.

Cloning, Characterisation and Transcriptional activity of the Human Calcitonin Receptor Promoter in Transgenic Mice

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The calcitonin receptor (CTR) is member of a newly identified subfamily of the seven-membrane domain G protein-coupled receptor superfamily. CTRs are highly expressed in primary breast cancer, kidney and in osteoclasts. Despite CTRs potential role in diseases such as osteoporosis and breast cancer little is known about the transcriptional regulation of the gene. To initiate studies on the transcriptional regulation of the human CTR (hCTR) gene, we have therefore cloned and characterised the human CTR promoter and studied its tissue and developmental specific transcriptional activity in transgenic mice. To identify transcriptional initiation sites in the hCTR promoter,we performed 5' RACE analysis on RNA from kidney, osteoclastomas and the breast cancer cell line T47Ds (all CTR positive). Multiple initiation sites were identified but all mapped close to the transcriptional initiation site previously mapped in the porcine CTR promoter. Additional RT-PCR identified transcripts initiating at least 1 kb upstream the mapped hCTR transcriptional initiation sites. This suggested that hCTR gene expression is regulated by multiple promoters. Preliminary results have now suggested that the downstream CTR promoter produces two CTR isoforms (C1A and C1B), while the upstream promoter expresses only one isoform (C1A).To study the transcriptional activity of the hCTR promoter in vivo, we generated transgenic mice containing the hCTR promoter linked to a lacZ reporter gene.The hCTR promoter was active as early as 8.5 days of mouse development. LacZ expression was first observed on the lateral half of somites. By 9.5 days of development, lacZ positive cells could be observed migrating to the limb buds and anterior body wall. By 15.5 days of development, limb and intercostal muscles were lacZ positive. It has previously been demonstrated that virtually all of the cells residing in the later half of newly formed somites are destined to leave the somite and populate the limb muscles and muscles of the ventral body wall. Our data therefore suggests that the hCTR promoter is first active in cells from a distinct myogenic lineage. This suggests CTRs may play an important role in muscle development. Further analysis identified novel sites of CTR expression such as the skin, cornea, retina and salivary gland, suggesting a role for the CTR in the morphogenesis of these tissues. Surprisingly, the hCTR promoter was not active in kidney or osteoclasts suggesting that additional sequences either 5' or 3' may be required for hCTR promoter activity in these tissues.

K-*ras* modulates DNA damage-induced apoptosis in embryonic stem cells

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Mutation of the K-*ras* oncogene is found in 40% colorectal neoplasms. A possible mechanism for the action of K-*ras* in neoplasia is by interference with cellular apoptotic pathways, as previous studies have implicated other members of the *ras* family in the suppression of tumour apoptosis.
To investigate the effects of K-*ras* on apoptosis *in vitro*, we constructed plasmid vectors to express both wild-type (gly^{12}) and mutant (val^{12}) K-*ras* in cultured cells. Murine embryonic stem (ES) cells were used for these studies as they represent a more normal genetic background compared to malignant cell lines which frequently have multiple mutations in cancer-related genes. Furthermore, in order to avoid the complications of endogenous K-*ras* expression, K-*ras* double knockout ES cells were generated by gene targeting. The wild type and valine mutant K-*ras* expressing plasmids were transfected into these K-*ras* null ES cells to generate stable cell lines.
ES cell lines (wild-type, valine-*ras* expressing clone, glycine-*ras* expressing clone and untransfected *ras*-null) were treated with either UV radiation, etoposide or cisplatin to induce DNA damage and hence apoptosis. No difference was found in the rates of apoptosis between the cell lines on exposure to UV. However, both etoposide and cisplatin were found to produce approximately 2 fold more apoptosis in the val^{12}-expressing clone, compared to the gly^{12}-expressing clone and wild-type ES cells. Furthermore, the incidence of apoptosis in the *ras*-null ES cells was found to be approximately half of that seen in the gly^{12}-expressing clone and wild-type ES cells after etoposide or cisplatin treatment.
These data suggest that, in this cellular context, mutant K-*ras* increases apoptosis in response to etoposide or cisplatin-induced DNA damage. This is consistent with evidence of *ras*-induced enhancement of apoptosis in lymphocytes. By contrast, expression of mutant H-*ras* in fibroblast tumours suppresses apoptosis. Differences in *ras*-mediated modulation of apoptosis may indicate H- or K-*ras* specific effects, cell type-specific differences in *ras* signalling pathways, or that *ras*-mediated suppression of apoptosis involves co-operation with other cancer gene mutations.

High Expression of HHV-8 Gene Products on AIDS-associated, but not on AIDS-unrelated Muticentric Castleman's disease

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Multicentric Castleman's Disease (MCD) is an uncurable chronic disease of prolonged course characterized by the severe polyclonal hypergammaglobulinemia, lymphadenopathy with characteristic pathomorphology such as hyaline vascular change, angiosclerosis or marked polyclonal plasmacytosis, and generaluzed symptoms such as malaise, fever, hepatosplenomegaly and occasional renal insufficiency. A part of MCDs are known to associate with AIDS. While its pathogenesis remains unclear, the association of human herpesvirus type 8 (HHV-8) has been stressed recently on a part of AIDS-associated and non-AIDS MCDs. However, prominent disaccordances were noted among reports of non-AIDS MCDs in terms of HHV-8 positivity. In the present study, we intended to separate MCDs associating and not associating with HHV-8 in a convincing way. First, we raised two rabbit antibodies that recognize HHV-8 latent gene (ORF59) product and lytic gene (ORF73) product specifically. Analysis was performed on pathology specimens of 61 cases with immuno-histology, in situ hybridization and genomic PCR. While there were three MCDs from AIDS patients and the remainings from HIV-negative (21cases) or HIV infection unclear (37 cases), immunostainings and in situ hybridization showed identical results that identical 3 cases were positively stained. Peculiarly, all these 3 cases were the AIDS cases. Genomic PCR studies was done on 43 cases, in which only one case represented constant positive results, and again, this case was the only one AIDS cases studied by PCR. Thus, it is suggested that the MCDs occurring in AIDS patients are highly associating with HHV-8, while the association of HHV8 is unlikely, or very much limited at most, on non-AIDS MCD cases.

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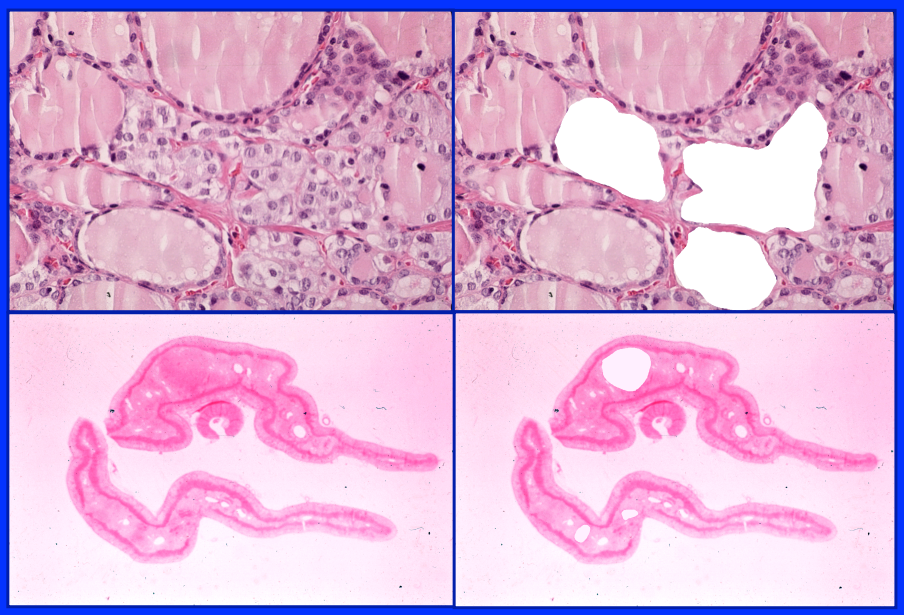
Early Monoclonal Expansions in MEN-2A

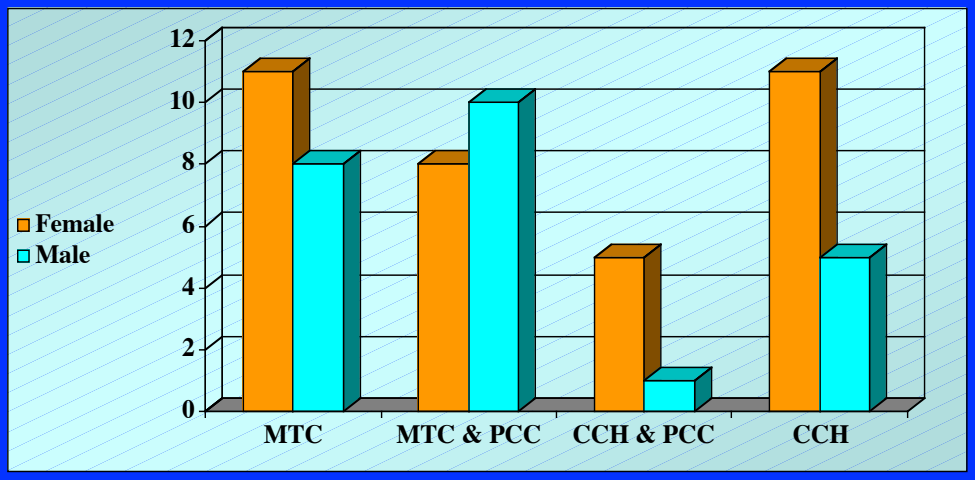
- Histologic definitions of C-Cell Hyperplasia (CCH) and Adrenal Medullary Hyperplasia (AMH) are controversial
- They normally show a multifocal growth
- The clonal pattern of those foci remains essentially unknown

Early Monoclonal Expansions in MEN-2A Methods

- Female patients with germline point mutation (*RET* codon 634): 10 bilateral CCH and 11 bilateral AMH
- HUMARA assay of clonality independently performed in each focus:
  - 20 CCH foci (1 per lobe and patient)
  - 34 AMH nodules < 1 cm in diameter
- Comparative analysis of the methylation pattern

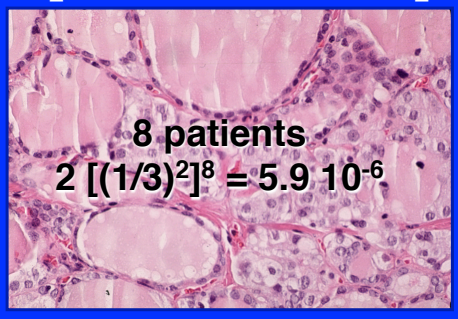


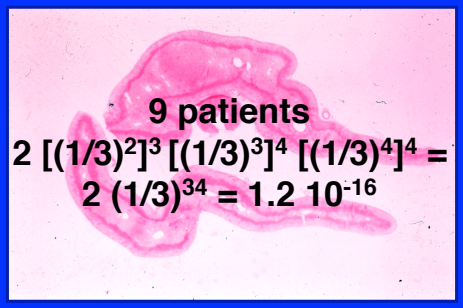
Early Monoclonal Expansions in MEN-2A Patients



Early Monoclonal Expansions in MEN-2A Results

p(AR pattern in tissue) = 1/3
No of AR Allele (informative patients) = 2
n = No of lesions compared
x = No of patients
p(concordant AR pattern) = $2\ [(1/3)^n]^x$





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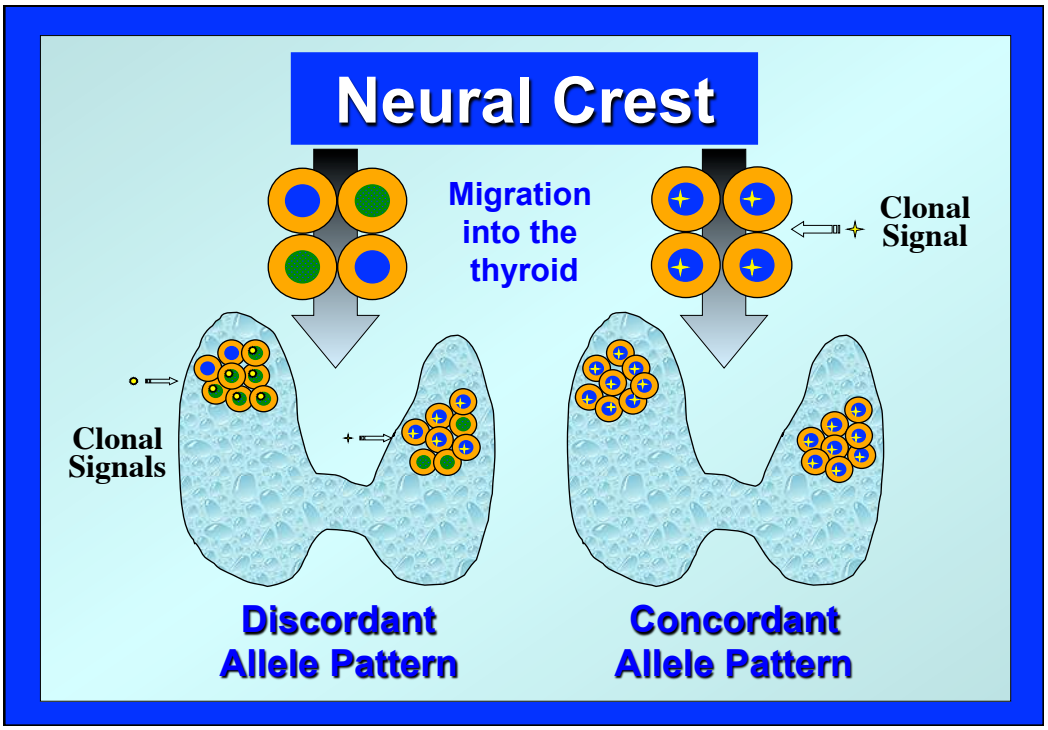
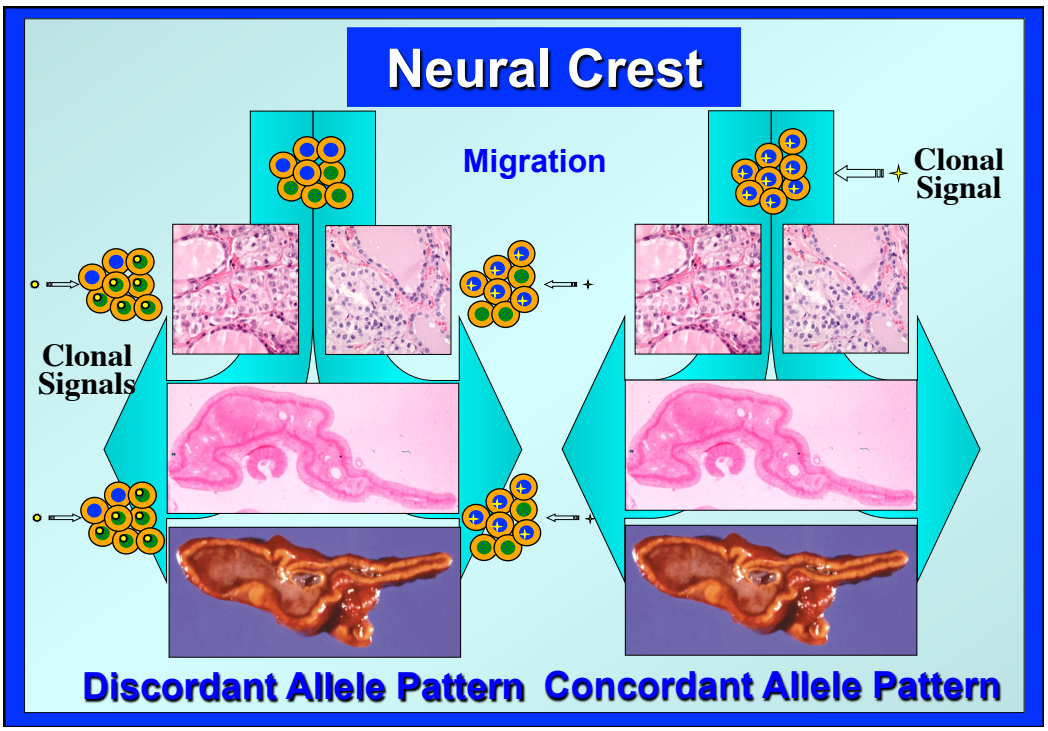
C-Cell Hyperplasias

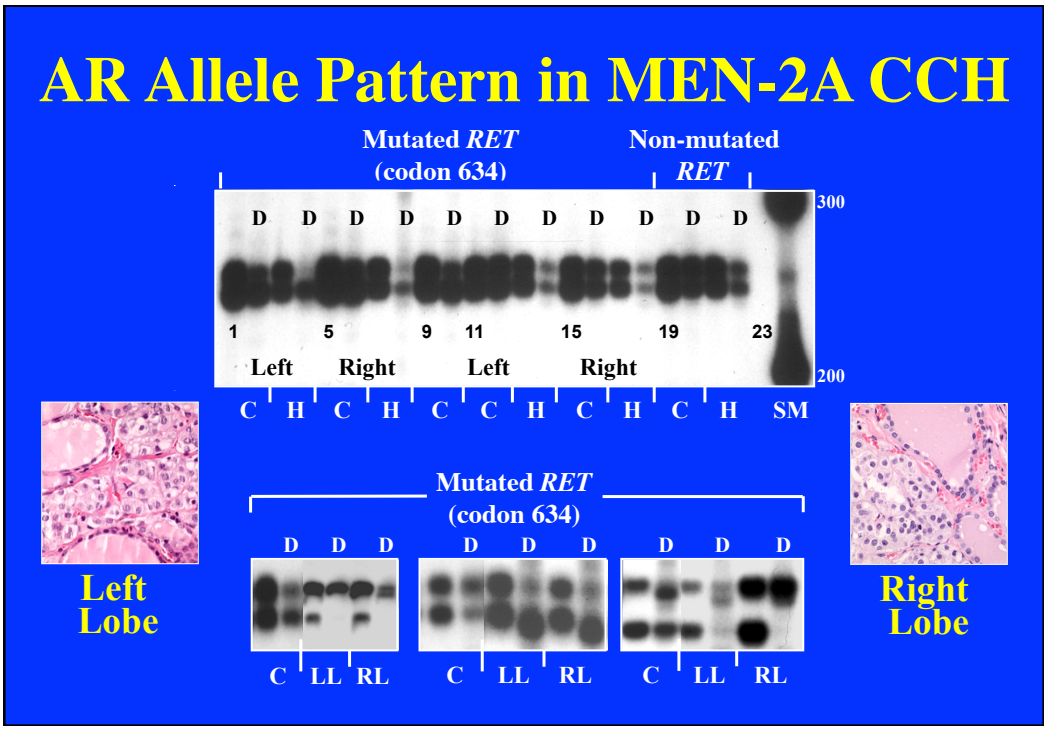
8 patients
$2\ [(1/3)^2]^8 = 5.9\ 10^{-6}$

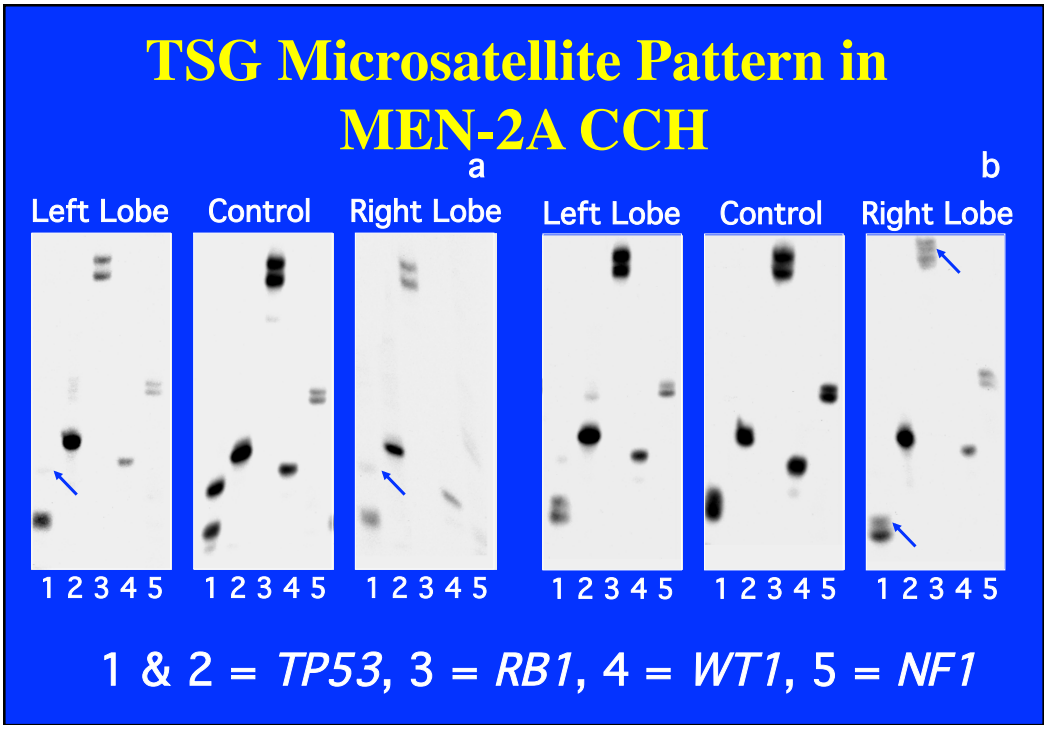
Adrenal Medullary Hyperplasia

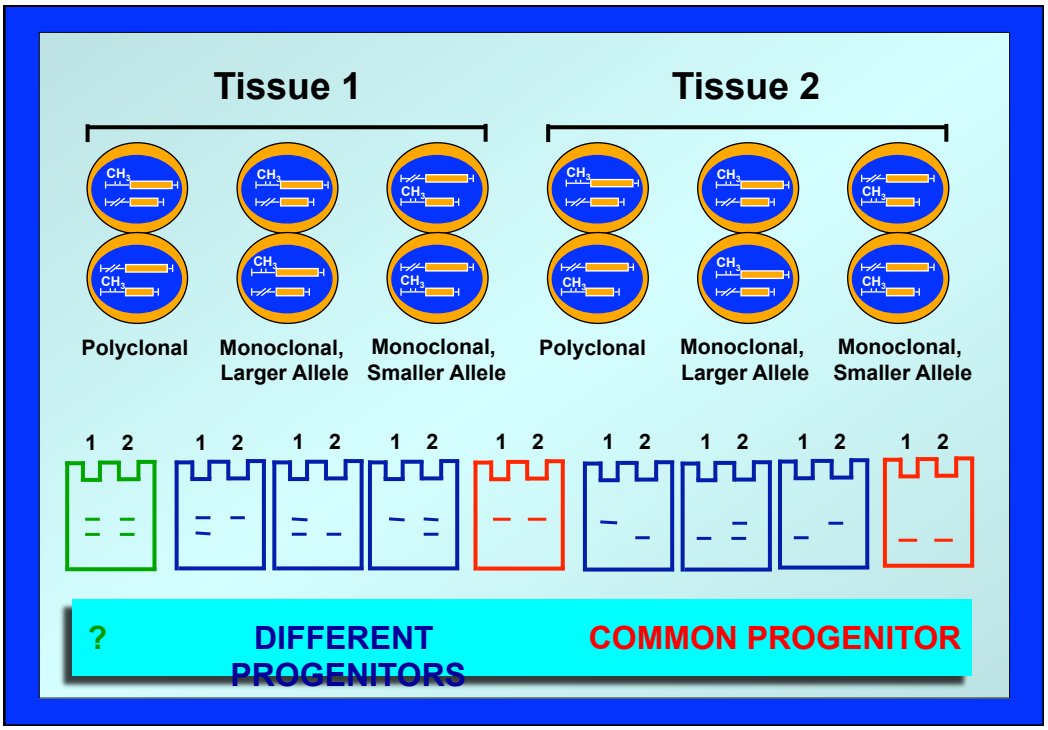
9 patients
$2\ [(1/3)^2]^3\ [(1/3)^3]^4\ [(1/3)^4]^4 =$
$2\ (1/3)^{34} = 1.2\ 10^{-16}$

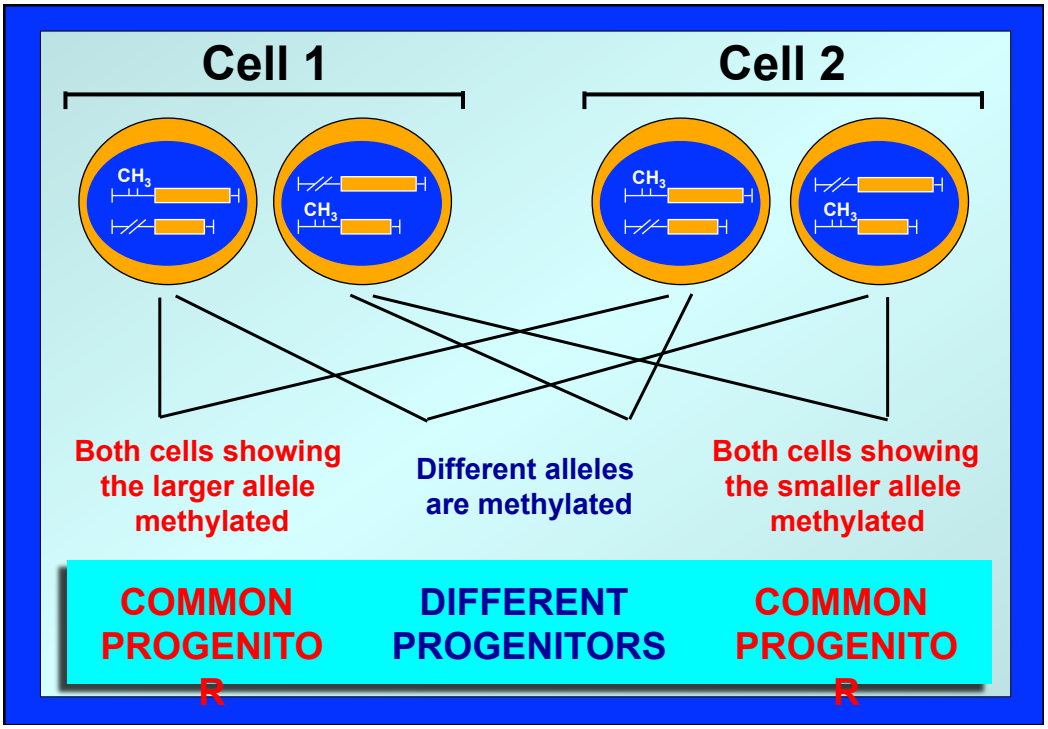


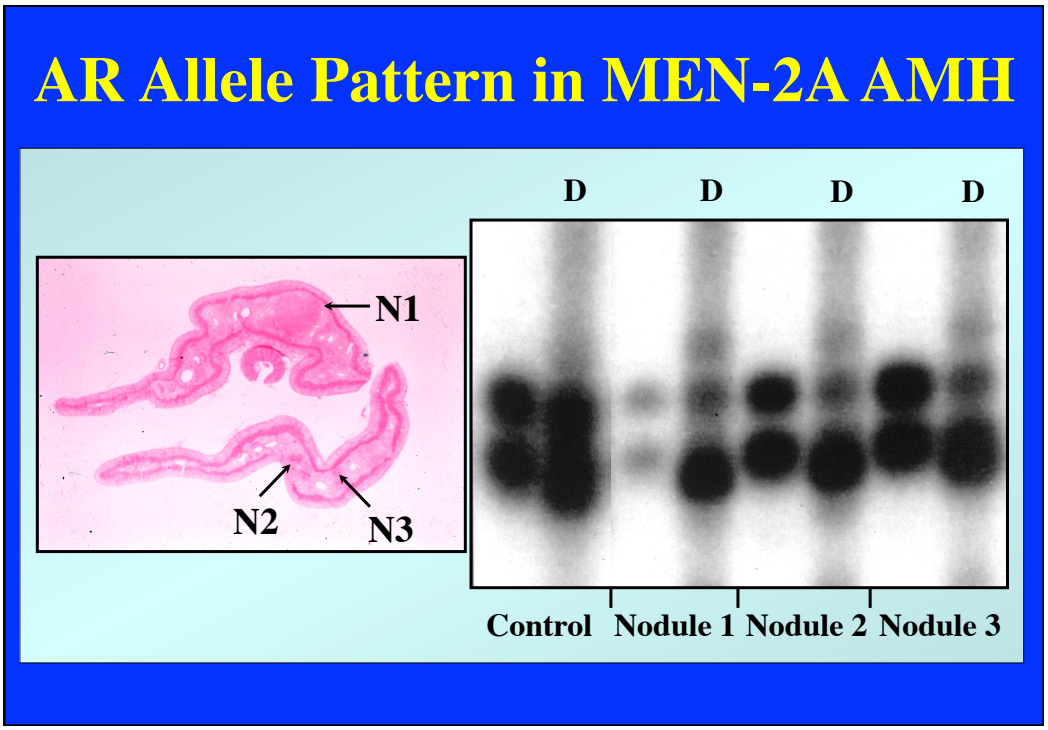

Neural Crest
Migration
Clonal Signal
Clonal Signals
Discordant Allele Pattern
Concordant Allele Pattern

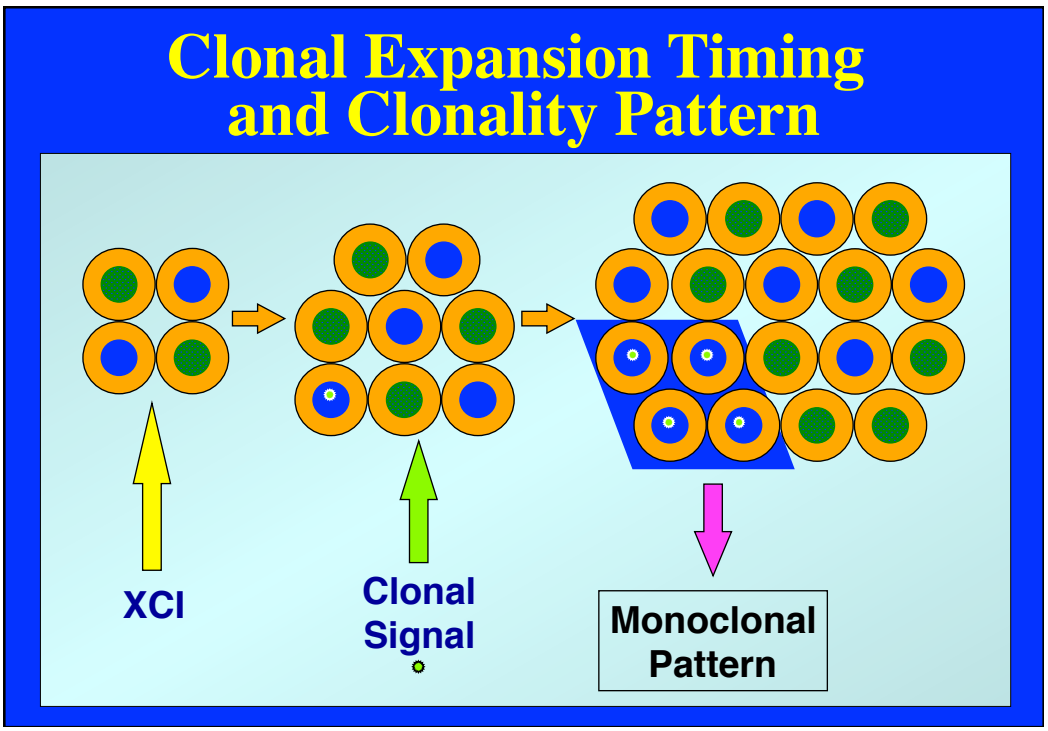

AR Allele Pattern in MEN-2A CCH
Mutated RET (codon 634)
Non-mutated RET
D D D D D D D D D D D
300
200
1 5 9 11 15 19 23
Left Right Left Right
C H C H C C H C H C H SM
Left Lobe
Right Lobe
Mutated RET (codon 634)
D D D D D D D D D
C LL RL C LL RL C LL RL

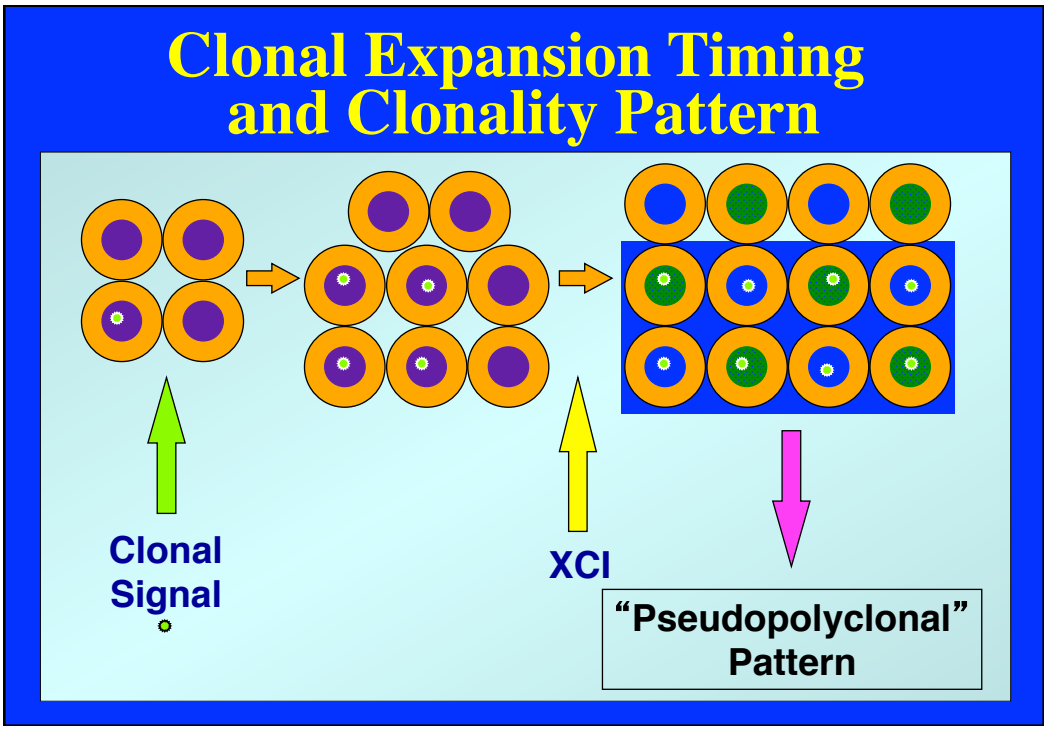

TSG Microsatellite Pattern in MEN-2A CCH
a
b
Left Lobe
Control
Right Lobe
Left Lobe
Control
Right Lobe
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 2 3 4 5
1 & 2 = TP53, 3 = RB1, 4 = WT1, 5 = NF1


Tissue 1
Tissue 2
Polyclonal
Monoclonal, Larger Allele
Monoclonal, Smaller Allele
Polyclonal
Monoclonal, Larger Allele
Monoclonal, Smaller Allele
1 2
?
DIFFERENT PROGENITORS
COMMON PROGENITOR


Cell 1
Cell 2
CH3
CH3
CH3
CH3
Both cells showing the larger allele methylated
Different alleles are methylated
Both cells showing the smaller allele methylated
COMMON PROGENITOR
DIFFERENT PROGENITORS
COMMON PROGENITOR


AR Allele Pattern in MEN-2A AMH
D
D
D
D
N1
N2
N3
Control
Nodule 1
Nodule 2
Nodule 3


Clonal Expansion Timing
and Clonality Pattern
XCI
Clonal
Signal
Monoclonal
Pattern


Clonal Expansion Timing
and Clonality Pattern
Clonal
Signal
XCI
“Pseudopolyclonal”
Pattern

Early Monoclonal Expansions in MEN-2A Conclusions

- CCH and AMH are mainly monoclonal proliferations with concordant methylation of androgen receptor alleles in a given MEN-2A patient (*RET* point mutation at codon 634)
- The multifocal and concordant nature of both conditions suggests an early clonal expansion of precursors at certain point