

RATS, MOLES AND VON RECKLINGHAUSEN

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VON RECKLINGHAUSEN'S DISEASE

Neurofibromatosis type 1 or von Recklinghausen's disease is one of the most striking of all genetic disorders and is characterised by skin lumps, café au lait patches, iris nodules, skinfold freckling, and deep-seated neural tumours⁽¹⁾.

Patients are usually normal at birth. Skin lumps (neurofibromas) start to develop in childhood and early adolescence⁽¹⁾, increase in number into old age, and can become extremely disfiguring (Figure 1). Café au lait patches are flat, lightly-pigmented areas of skin, six or more of which are almost diagnostic of neurofibromatosis⁽²⁾. Most patients also have freckles in axillae and groins. Iris lesions are pigmented hamartomas known as Lisch nodules⁽³⁾. Deep nerves can be enormously expanded by plexiform neurofibromas causing considerable morbidity⁽⁴⁾.



Figure 1 neurofibromatosis

Similar conditions have been reported in historical literature⁽⁵⁾ as far back as the thirteenth century. In 1847 Virchow described a family with cutaneous neuromas. Friedrich von Recklinghausen, who at the age of 22 had become Virchow's assistant, made a further description of the condition in 1882 and coined the term "neurofibroma", dedicating his paper to Virchow on the 25th anniversary of the Pathological Institute of Berlin. It is now realised that neurofibromatosis comprises two separate syndromes; neurofibromatosis type 1 (described above) and neurofibromatosis type 2 which is much rarer and dominated by deep-seated tumours, particularly acoustic neuromas^(3,5).

Neurofibromatosis type 1 is also one of the commoner genetic diseases, its incidence approaching that of cystic fibrosis (Figure 2). By "neurofibromatosis" we shall refer in this article to neurofibromatosis type 1.

The defective gene in neurofibromatosis⁽³⁾ is on chromosome 17. The gene has one of the highest mutation

Disease	Incidence	Inheritance
Polycystic kidneys	1/1250	Autosomal dominant
Huntington's chorea	1/2000	Autosomal dominant
Cystic fibrosis	1/2000	Autosomal recessive
Neurofibromatosis type 1	1/3000	Autosomal dominant
Duchenne muscular dystrophy	1/4000	X-linked
Neurofibromatosis type 2	1/33000	Autosomal dominant

Figure 2 Neurofibromatosis compared with other common genetic diseases^(3,6). Incidence per live births

rates known in humans and about 50 percent of cases of neurofibromatosis represent new mutations⁽⁶⁾. The gene codes for the synthesis of a protein **neurofibromin** which restrains cell proliferation by inhibiting **ras**⁽⁷⁾.

RAS

The term "**ras**" is derived from "rat sarcoma virus". This virus was discovered almost by accident in 1964 by Jennifer Harvey at Northwick Park⁽⁸⁾. Rats were infected at birth with Moloney leukaemia virus which is a slow-transforming RNA virus composed of just three genes (Figure 3). A DNA copy of these genes is now known to be integrated into the host cell genome⁽⁹⁾ and leads eventually to leukaemia in a proportion of cases. From one of the resulting leukaemic rats, filtrates of diluted plasma were found to contain an extremely potent cancer-producing agent, rat sarcoma virus. This produced sarcomas in rats only 12 days after inoculation and killed the animals within a month⁽⁸⁾.

gag	pol	env
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Figure 3 The three genes of the slow-transforming Moloney leukaemia RNA virus. **gag** codes for viral internal proteins, **pol** for reverse transcriptase, **env** for viral envelope⁽⁹⁾

Rat sarcoma virus is a fast-transforming RNA virus composed of three genes, one of which is an oncogene **ras** (Figure 4). The virus probably evolved from the leukaemia virus by substitution of the viral **pol** gene by a **ras** gene derived from the host animal⁽⁹⁾. Having lost the **pol** gene, however, rat sarcoma virus needs the help of a concurrent RNA virus infection to replicate itself i.e. it has become a defective virus. Although first found in rat sarcoma virus, the **ras** gene seems to be ubiquitous and an essential component of eukaryotic cells from the tiny soil nematode worm (*Caenorhabditis elegans*)⁽¹⁰⁾ to humans.

gag	ras	env
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Figure 4 The three genes of the fast-transforming rat sarcoma virus. **Ras** is an oncogene derived from a host cell

The **ras** gene codes for a protein which is a central part of the signalling pathway leading to cell division (Figure 5). **Ras** protein is anchored to the inner surface of the cell

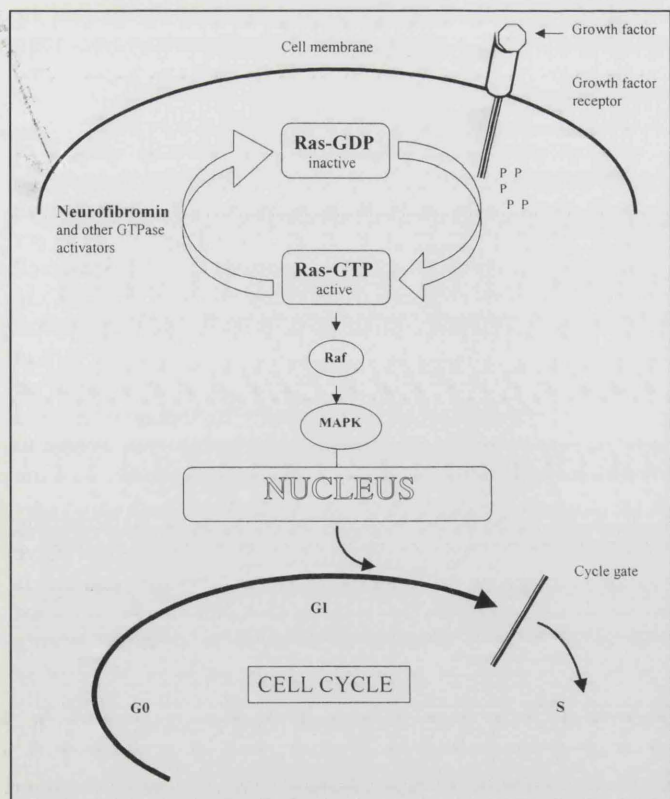


Figure 5 Ras signalling pathway

MAPK = mitogen-activated protein kinase

P = phosphate groups

Cycle gate = restriction point

G0, G1 and S are phases of the mitotic cycle⁽¹³⁾

membrane⁽¹¹⁾ and is combined with either guanine diphosphate (GDP) or guanine triphosphate (GTP). Here **ras** is continually cycling between an inactive form, **ras-GDP**, and an active form, **ras-GTP**. **Ras-GTP** activates the signalling cascade where successive components are phosphorylated ending in MAPK (mitogen-activated protein kinase). MAPK can then enter the nucleus to modify the transcription of genes involved in cell division^(7,12).

As a result of the signal, cells leave a resting state (G0), enter the mitotic cycle and progress through the G1 phase as far the cycle gate ("restriction point") where further controls are effected⁽¹³⁾ before cells are permitted to commence DNA replication of the S phase⁽¹⁴⁾.

The mitogenic signalling pathway is initiated when growth factor receptors in the cell membrane combine with their specific growth factor in the extracellular fluid^(7,12). Growth factor receptors are transmembrane proteins. When the extracellular component receives its growth factor, the intracellular component causes **ras-GDP** to be phosphorylated and thereby activated.

Ras itself slowly hydrolyses GTP to GDP and therefore gradually inactivates itself, behaving as a GTPase enzyme. Neurofibromin is one of a number of negative-regulators of **ras** which rapidly hydrolyse **ras-GTP** to **ras-GDP**. In the absence of neurofibromin there is accumulation of **ras-GTP** and excessive mitotic drive.

RAS AND CANCER

Ras genes are commonly found to be mutated in human cancers. A single point-mutation can be sufficient to render **ras-GTP** resistant to GTPases such as neurofibromin, again leading to accumulation of active **ras-GTP** and excessive

mitotic drive⁽⁷⁾. This type of mutation is found in 90% of pancreatic carcinomas, 50% of colorectal carcinomas as well as many other tumour types⁽¹⁵⁾. Mutant **ras** or missing neurofibromin do not by themselves lead to cancer. After all cancer is a multistep process and an activated **ras** pathway drives cells up to the mitotic control gate but not necessarily through it⁽¹⁴⁾. Cancer also requires the gate to be wedged open and cells to have unlimited mitotic potential. Recently human cells have been converted into cancer in a culture dish. A minimum of four separate mutations were required⁽¹⁶⁾.

- Telomerase has to be activated (allowing unlimited mitoses)
- **Ras** has to be permanently activated
- P53 has to be activated⁽¹³⁾
- Retinoblastoma protein has to be inactivated⁽¹³⁾

Malignant transformation of neurofibromas, mostly of the large plexiform lesions, is a rare complication of neurofibromatosis, affecting about 2% of cases⁽³⁾. The process is a result of the accumulation of further specific mutations in the tumour cells. Neurofibrosarcoma is extremely rare in normal individuals because acquisition of the full set of mutations, without a head-start, is highly improbable.

NEUROFIBROMAS

Patients with neurofibromatosis are usually normal at birth. At this stage all their cells will contain a single functional neurofibromin-producing gene and will behave normally. It is only when the one remaining gene is lost by mutation (the second hit) that **ras** is no longer controlled and a neurofibroma develops^(17,18). The process seems to target the specialised fibroblasts in nerve sheaths and will be a random event related to mitosis. The mutation rate (for non-germline cells) has been calculated to be 1 in 10 million per gene per cell generation⁽¹⁹⁾. The numbers and proliferative activity of these specialised fibroblasts is unknown, but each neurofibroma marks the site where a single fibroblast sustained a mutational hit on its one remaining functional neurofibromin gene.

Patients are born normal because their specialised fibroblasts have not yet passed through sufficient mitoses (cell generations) to make the specific mutation probable.

Can we learn anything from neurofibromatosis? Here we have a condition in which patients start off apparently normal but then gradually develop pigmented skin patches and nodules, now known to be due to the acquisition of single mutations at a particular gene site. Could common pigmented melanocytic naevi (moles) also be the result of a single dominant genetic mutation?

MOLES

It has never been clear whether moles are malformations or tumours, but most authors consider moles to be benign neoplasms⁽¹⁾. The rest of us are probably too busy diagnosing and removing moles to ponder their pathogenesis for long. For tumours, however, moles are far too common and precocious; unlike malformations, moles are rarely present at birth.

Most people are born mole-free. Only about 1% of newborn infants are found to have a mole (congenital melanocytic naevus)⁽²⁰⁾. Moles usually appear in childhood and adolescence and reach a maximum at about 25 years of age^(1,21). The average

population of cells	doubling cycle (completed) = x	mutated cells at rate (m) = 2×10^{-8}		location
		new	cumulated total	
2.1×10^9	31		44	epidermal melanocytes, after birth
1.1×10^9	30		22	
5.4×10^8	29		12	
2.7×10^8	28		6.2	
1.3×10^8	27		3.6	epidermal melanocytes, pre-birth
6.7×10^7	26		2.0	
3.4×10^7	25		1.4	
4.2×10^6	22		0.8	
2.1×10^6	21		0.8	
1.0×10^6	20		0.8	
1.0×10^6	20	0.4	0.77	neural crest: per-melanocytes
3.3×10^4	15	9.9×10^{-3}		
1024	10	2.0×10^{-4}		
32	5	3.2×10^{-6}	c	
1.0	0		4.0×10^{-7}	b
↑ DEVELOPMENT OF THE NEURAL CREST FROM A SINGLE CELL				
1.0×10^6	20		0.4	a
1024	10			embryo: per-neural crest
zygote	0			

Figure 6 Adult population of melanocytes derived by sequential doubling cycles from zygote. Some cycles and calculations omitted for brevity. Mutant cells expected, given a mutation rate of 1×10^{-8} per cell per generation per gene (rate is doubled for two alleles)

number of moles on a young adult is estimated at between 10 and $40^{(21,22)}$. Thereafter moles gradually decline and fade, becoming uncommon in old age^(1,21).

MELANOCYTES

Much more is known about melanocytes than neural fibroblasts. Cutaneous melanocytes arise in the neural crest, migrate to the skin, and colonize the epidermis from the eighth week of gestation⁽²¹⁾. The neural crest is a short-lived embryonic structure which gives rise to a range of different cell types including neural tissue, some cranial connective tissue, adrenal medulla and melanocytes⁽²³⁾. The neural crest soon undergoes subdivision into several anatomical parts, but its proliferating cells remain pluripotent during most of this phase, and final determination of their differentiation may even occur during their migration⁽²³⁾.

In the skin melanocytes settle in the basal layer of the epidermis (including hair follicles) and are normally separated from each other by about six basal epidermal cells^(1,21). Each melanocyte supplies pigment to approximately 36 adjacent keratinocytes, forming an "epidermal melanin unit"⁽²⁴⁾. Within the melanocyte the enzyme tyrosinase converts tyrosine to dopa and then dopaquinone, which is then oxidised to melanin⁽²⁴⁾. Melanocytes have long dendritic cytoplasmic processes through which melanin is transported to the keratinocytes⁽²⁵⁾.

The population of cutaneous melanocytes in an adult male has been estimated at 2×10^9 cells^(21,26). At birth there are about 800 melanocytes/mm² of skin, a concentration which is maintained in adults and all races⁽²⁷⁾. Melanocytes in skin can continue to reproduce themselves by mitosis but this is rarely observed⁽²¹⁾, unlike mitoses in other epidermal cells. By the age of forty, cutaneous melanocytes seem to become

senescent and their density gradually declines throughout the rest of life, at 6-8% per decade⁽²¹⁾. Loss of melanocytes from hair follicles is the cause of the all too familiar age-related greying of hair⁽²⁷⁾.

When normal cells divide there is a small chance of genetic mutation due to errors in DNA replication or subsequent phases of mitosis. Spontaneous mutation rates in somatic cells is thought to be about 1 in 10 million cells per generation per gene, usually expressed as 10^{-7} /gene/cell generation⁽¹⁹⁾, but estimates have ranged from 5×10^{-8} to 5×10^{-6} /gene/cell generation⁽²⁸⁾. A recent study of human fibroblasts has demonstrated a spontaneous mutation rate in the range 3×10^{-8} to 5×10^{-7} ⁽²⁹⁾. Amazing as it may seem, we can estimate the number of mutations in melanocytes and their precursors by tracing their development from zygote to adult skin (Figure 6).

A population of 2×10^9 cells will require 31 doubling cycles for its generation from a single stem cell. I have assumed somewhat arbitrarily that the first neural crest cell is twenty divisions from the zygote, and that the neural crest cells expand from a single stem cell for a further twenty divisions to produce one million pre-melanocytes (lower shaded zone in figure). This number of cells incidentally would weigh about 1 milligram⁽³⁰⁾. I have assumed that the cells migrate at this stage and colonise the fetal skin. From birth to adulthood skin surface area increases about 7.4-fold⁽³¹⁾. This implies that the last three doubling cycles of melanocytes occur after birth i.e. cycles 28-31 (upper shaded zone in figure). The methods for calculating mutations (a to e in Figure 6) are different in three compartments, pre-neural crest, neural crest, and post-neural crest; formulae are given in the appendix.

MOLES ARE DUE TO A SINGLE MUTATION

The hypothesis is that a mutant melanocyte will grow into a mole. Several observations can now be made from the model:

- Almost all the mutations occur in the last few cycles i.e. after birth
- A mutation rate of 10^{-8} generates a realistic number of moles in an adult
- Congenital moles will be rare. In this particular model, six mutant melanocytes are somewhere in the skin at birth but these may not yet have grown into a visible mole.

Involvement of more than one mutated gene is impossible as moles would be much too rare. Even taking a high rate for gene mutation (10^{-6}), the probability of two independent specific "hits" would be 10^{-12} and a mole would only occur at most in 1% of adults, and more likely would be a rare curiosity just like sporadic neurofibromas.

For an infant to be born with a mole, a mutant melanocyte has not only to occur but have time to proliferate sufficiently to become visible by birth. The further back in melanocyte development that a mutant appears the larger will be the resulting congenital mole, as more doubling cycles can be completed by the mutant cell before birth. On the other hand mutants become more and more improbable as the population of melanocytes is traced back to its stem cell. So we can predict from our model the observation that the larger a congenital mole, the rarer it is. Some giant hairy naevi probably originate from mutations in the neural crest cells.

A single mutation, then, could explain much of how moles develop. A mutational hypothesis is the only way of explaining the age-pattern of onset of moles. The final few doubling cycles after birth are sufficient to bring out most of

the mutations due to the large population of dividing cells. Moles stop coming in adulthood because the skin is no longer expanding in area and melanocytes are already approaching their age of retirement. A mutational hypothesis would also explain the observation that sun exposure and maintenance chemotherapy cause excessive numbers of moles to develop^(21,32).

The cellular effect of the "mole mutation" can only be guessed at. Melanocytes which form moles differ from normal melanocytes in several ways⁽²¹⁾. Melanocytes have long dendritic cytoplasmic processes, keep well spaced out in the epidermis, and have strictly controlled growth. Mole cells are rounded, cluster together, and keep growing to form nodules. Cell to cell contact and cell adhesion to a substrate are known to activate signalling pathways which can control cell proliferation^(33,34). Perhaps in moles cell contact fails to suppress growth.

It is just possible that the **ras** gene itself is the target for the mole mutation. A recent study has identified a high incidence of **ras** mutations in congenital melanocytic naevi (moles)⁽³⁵⁾. The **ras** gene probably presents a relatively small target for mutation as it only takes one of a few point mutations to fix **ras** in its active form⁽⁷⁾. The mole mutation rate in the model is indeed rather low. A **ras** gene target could also explain why a single mutation might affect a cell; the mutant allele could still increase the cell content of activated **ras**. So are moles the result of a single (heterozygous) **ras** mutation?

Moles are, however, almost invariably benign. Just as in the neurofibroma, malignancy requires more highly-probable mutational damage.

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APPENDIX

Calculation	Formula	Reference
a	mx	28
b	mx/1.0 x 10 ⁶	28
c	mx2 ^x	28
d	Σmx2 ^x for x = 0 to x = 20	28
e	[(2 ²¹ -1)-(2 ²⁰ -1)]m	Derived from sum of geometric series

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