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A study of anticipation in families with hereditary non-polyposis colorectal cancer (HNPCC)

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Abbreviations

HNPCC	-	Hereditary non-polyposis colorectal cancer
CRC	-	Colorectal cancer
FAP	-	Familial adenomatous polyposis
MSI	-	Microsatellite instability
AC	-	Amsterdam criteria
ICG-HNPCC	-	International Collaborative Group of HNPCC
ACII	-	Amsterdam II criteria
BC	-	Bethesda criteria
MMR	-	Mismatch-repair
hMSH2	-	human MutS Homologue 2
hMSH6	-	human MutS Homologue 6
hMLH1	-	human MutL Homologue 1
IHC	-	Immunohistochemistry
CI	-	Confidence interval

Summary

The phenomenon of anticipation, earlier onset in successive generations, occurring in hereditary non-polyposis colorectal cancer (HNPCC), has been both supported and rejected in previous publications. Using a different approach than previous studies, 83 families with a total number of 298 individuals from the Karolinska University Hospital were studied. The effect of anticipation was calculated using two different models. Both methods were based on calculating the difference between the previous and next generation. After statistical analysis, using the cluster method, it was shown that children developed tumors 8 years earlier than their parents. When using the same patients but this time calculating the difference with the individual method the next generation again showed almost 8 years earlier age of onset than the previous generation. Since HNPCC is related to defects in three mismatch repair genes (*hMLH1*, *hMSH2* and *hMSH6*), the relative contribution to anticipation was calculated for every gene. *hMLH1* showed 7.5 years earlier onset in the next generation compared to the previous generation, *hMSH2* showed approximately 12 years and *hMSH6* showed 5.5 years. In other words, it was unmistakably found that anticipation occurs in HNPCC families.

Introduction

Earlier age of onset in successive generations, or anticipation, was first documented in the early 20th century, but was primarily considered as a result of ascertainment bias (Mott 1911). An influence of anticipation has been suggested mainly in cases of neurodegenerative diseases, due to their genetic composition, but anticipation has been observed also in cancers, diabetes, schizophrenia and dementia. Anticipation in cases of cancerous diseases was found in the 1940's in families with hereditary breast cancer and since then it has been demonstrated in familial leukemia, ovarian cancer, pancreatic cancer etc. (Jacobsen 1945, Horowitz *et al.* 1996, Goldberg *et al.* 1997, McFaul *et al.* 2006). The effect from anticipation in hereditary colorectal cancer has been tested in a small number of studies but the results have provided conflicting answers. Thus a study taking a different approach to this issue is highly relevant.

Cancer

Cancer is not just a common disease among all human populations around the world, but it has been observed in almost all vertebrates. The oldest evidence of cancer has been found in skeletons of dinosaurs (Rothschild *et al.* 2003). The term cancer is a broad description of hundreds of different diseases that are characterized by cells with abnormal growth patterns. Even though this is imprecise, scientists agree that cancer always is a genetic disorder. Regardless of the inducing factor, mutations are inherited through generations or from cell to cell. The mutations can then change the activity of proteins that are associated with regulatory stages in the cell cycle and cause a modified cell (Ruccione and Kelly 2000). In the last decade studies have shown that genes in the tumor generating pathways mainly are involved in angiogenesis (growth of new blood vessels), cell cycle, maintenance of the genome (DNA repair) and cell-cell signal transmission (Kinzler and Vogelstein 1996, Folkman 1996, Weinberg 1996). All these genes can be classified in three groups: tumor suppressor genes, oncogenes and DNA repair genes.

Tumor suppressor genes

The first evidence of tumor suppressor genes, genes whose products prevent cancer, was initially found when experimenting with fusions of tumor and non-tumor (somatic) cells. When fused together, in a culture, the hybrids that were formed did not generate cancer (Klein 1987). However, a fraction of the hybrid cells reverted back to a tumor generating state. This outcome was found to be linked to loss of chromosomes inherited from the non-tumor cell. In this study loss of a specific human chromosome was coupled to tumorigenic reversion (Geiser *et al.* 1986). It was proposed that a chromosome or even a single gene maybe would be enough to inhibit tumor development. This hypothesis was supported in several studies by suppressing tumorigenic growth of human cancer cells in nude mice (Saxon *et al.* 1986, Shimizu *et al.* 1990, Trent *et al.* 1990, Oshimura *et al.* 1990).

Oncogenes

The first indication of genes able to cause tumors, called oncogenes, was originally observed in rats, mice and chickens with transplanted tumors (Rous 1911). The active component in the tumor was found to be an RNA virus carrying a virus oncogene (v-onc). The oncogenes transmitted by the viruses are homologous to "normal" genes in the genome called proto-oncogenes (Kuff *et al.* 1981). Proto-oncogenes can be activated by several different mechanisms, for instance by insertion of a viral DNA at a proper position in the host DNA

(Tabin *et al.* 1982). Such insertions can modify the gene directly or affect its expression by modifying regulatory elements. It has also been shown that point mutations in proto-oncogenes cause changes in activity, for example in the *K-ras* gene, which often is discovered in colorectal tumors (Shibata *et al.* 1993).

DNA repair genes

The third group of genes involved in tumor development is the DNA repair genes, which are responsible for the process of maintaining the genomic information (Howard-Flanders and Boyce 1966, Pawsey *et al.* 1979). Inactivation or mutation does not directly encourage tumor development, but it leads to genetic instability that enhances the frequency of mutation in other genes, such as tumor suppressor genes. In humans the mismatch repair genes are functional in the postreplicative state of DNA repair. When inherited, the mutated alleles are recessive, this means that both the paternal and maternal alleles need to be dysfunctional in order to obtain a cell with an alternate phenotype. In hereditary condition, where a mutated allele is passed on to the offspring, a second mutation in the corresponding functional allele, due to e.g. a somatic mutation, will cause loss of heterozygosity. Genes responsible for tumor development in breast, *BRCA1* and *BRCA2*, have been found and it has also been suggested that mutations in germline mismatch repair genes are coupled to hereditary non-polyposis colorectal cancer (HNPCC) (Sharan and Bradley 1997, Scully *et al.* 1997, Kinzler and Vogelstein 1996).

Colorectal cancer

Colorectal cancer (CRC), cancer in the colon and rectum, is found and diagnosed all over the world and is actually the third most common form of cancer (Parkin *et al.* 2005). Men and women have a similar risk of developing a tumor, 1.2:1, respectively (Parkin *et al.* 2005). In Sweden on the average 5600 new cases of colorectal cancer were diagnosed between 2002 and 2006, 2800 in males and 2600 in females (Engholm *et al.* 2008). The incidence rates show small differences between sexes but the distribution across the world suggests a higher frequency of colorectal cancer in the western world (Parkin *et al.* 2005). It has also been shown that the incidence among immigrates can adapt quickly, sometimes within one generation, to the level in the host country (Heanzel 1961, McMichael *et al.* 1980, Stirbu *et al.* 2006). The international variations and recent changes in incidence rate in the eastern world indicate that CRC is influenced by changes related to the diet and other environmental conditions (Potter 1999). Studies have even shown that physical activity together with other factors can have a positive effect (Potter 1999). Colorectal cancer is a very complex disorder, as most cases of cancer, and there are many risk factors that influence the probability of developing a tumor. The most important one is family history, which indicates an inheritance component. The relative risk when having one affected first-degree relative (parent, child or sibling) is twice as high compared to the risk for an individual with healthy family background.

Hereditary non-polyposis colorectal cancer (HNPCC)

Many different factors can contribute to the development of colorectal cancer, and several of them are genetic. For instance, familial adenomatous polyposis (FAP) is a dominantly inherited autosomal disorder characterized by the development of hundreds to thousands of adenomatous polyps in the colon and rectum. Almost 100 % of the individuals carrying the mutation also express the related phenotype, thus the risk of developing colorectal cancer if untreated, no preventive measures, is extreme (Bisgaard *et al.* 1994). Another dominantly

inherited autosomal form of hereditary colorectal cancer is a syndrome called HNPCC, which accounts for 2-8 % of all cases (Burt 2000, Lynch *et al.* 2006). An overview of classes of colorectal cancer is shown in figure 1.

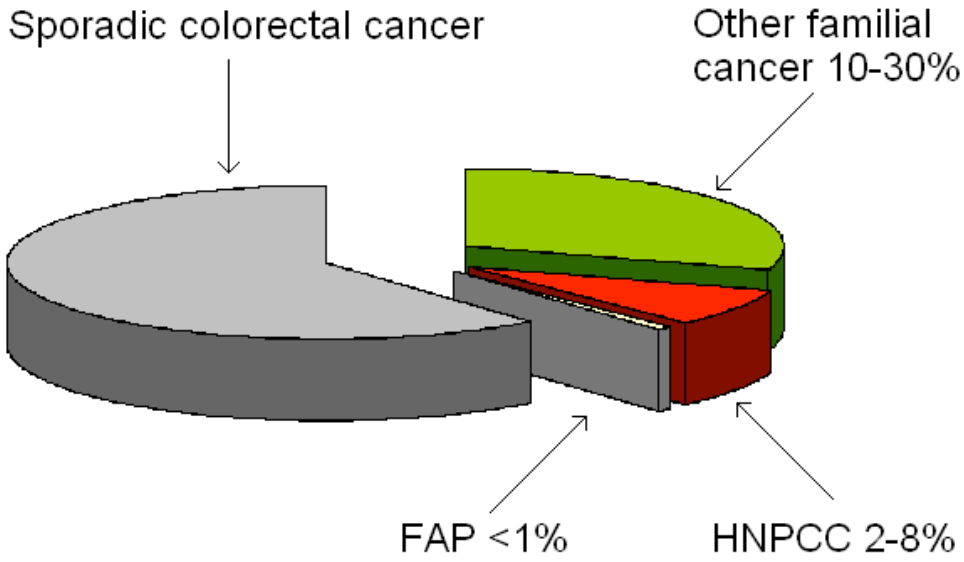

Figure 1. The different classes of colorectal cancer (data from Burt 2000). FAP = familial adenomatous polyposis; HNPCC = hereditary non-polyposis colorectal cancer.

HNPCC, also called Lynch syndrome, was first observed by Alfred Warthin who studied a family called family G that included a large number of individuals with gastrointestinal and endometrial cancers (Warthin 1913). Later Henry Lynch established the features of HNPCC by describing two additional families together with family G that showed an autosomal dominantly inherited pattern of colorectal cancer (Lynch *et al.* 1966 and Lynch and Krush 1971). In addition to colorectal and endometrial cancer, other HNPCC-associated tumors are also included in the disease profile, for instance cancers of the ovaries, stomach, small bowel, brain, liver, biliary tracts, urethra, ureter, bladder and kidneys (Aarnio *et al.* 1995, Aarnio *et al.* 1999, Vasen *et al.* 1990, Watson *et al.* 1993, Watson *et al.* 1994). Individuals with HNPCC develop a small number of adenomatous polyps that quickly can evolve into cancer at an early age.

It was mentioned earlier that HNPCC is caused by mutations in the DNA mismatch repair system. In tumors these mutation cause microsatellite instability (MSI), decreasing or increasing numbers of repetitions of specific DNA sequences. MSI is known as a distinct feature of HNPCC but it has been demonstrated also in around 15 % of all sporadic cases of colorectal cancer (Aaltonen *et al.* 1993, Ionov *et al.* 1993). Tandem repeats generally, in all cells, cause slippage of the polymerase during replication of the DNA, thereby inducing MSI. In normal cells these mistakes are repaired. Mutations in the DNA mismatch repair system prevent repair so that the errors remain. This in turn leads to alleles of different size (Hoang *et al.* 1997, Peltomaki 2001). Another hallmark of HNPCC is its relatively high penetrance, the percentage of individuals carrying the genetic mutation that also express the related phenotype. For mutations in *hMLH1* and *hMLH2* genes (described in more detail below) the penetrance is approximately 80 % by 75 years of age (Vasen *et al.* 1996).

Genes involved in DNA mismatch-repair

In 1993-1995 the three most familiar genes, *hMLH1*, *hMSH2*, and *hMSH6*, responsible for the function of the human DNA mismatch-repair (MMR) system were found (Fishel *et al.* 1993, Lindblom *et al.* 1993, Drummond *et al.* 1995). Ever since their discovery, these genes have been associated with HNPCC. Three additional MMR genes, *PMS2*, *MLH3* and *PMS1*, whose importance is not totally clear, also have been associated with HNPCC (Peltomaki 2005). Besides the mismatch repair of the post-replicative DNA, the MMR gene products are involved in a number of other essential functions in the cell, including various steps in apoptosis (Fishel 2001). Altogether nine MMR genes are known today, but three of them, *hMLH1*, *hMSH2* and *hMSH6*, account for more than 95 % of all identified HNPCC-related mutations (Peltomaki and Vasen 2004). The gene products are also a part of the signaling pathways that regulate programmed cell death. Thus a mutation that causes a loss of function will not only increase the mutation rate but also cause an abnormal cell growth that might result in a tumor (Fishel 2001). The major human MMR genes are described in more detail below.

hMSH2 (human MutS Homologue 2) is the human homologue to the *mutS* gene in bacteria and similar to MutS, it's product is responsible for binding to the mismatched site (Fishel *et al.* 1993). About 200 different mutations associated with Lynch syndrome have been found in MSH2 (Peltomaki and Vasen 2004). The main human mismatch-binding factor, hMutSα, has been found to consist of two proteins, hMSH2 and hMSH6 (human MutS Homologue 6), where the hMSH6 protein subunit is responsible for recognition of the mutated site (Drummond *et al.* 1995, Palombo *et al.* 1995, Iaccarino *et al.* 1998).

The third gene, *hMLH1* (human MutL Homologue 1), was found by analysis of Swedish families and with roughly 250 found mutations. It is said to be the most important gene of all HNPCC-related genes (Lindblom *et al.* 1993, Peltomaki and Vasen 2004). The function of hMLH1 includes recognition of mismatch linked with downstream steps in the MMR. In evolution, MMR proteins are conserved making it possible to make comparison between MMR in *Escherichia coli* and in humans.

The epigenetic effect

Tumorigenesis is often coupled to various genetic mechanisms that cause damaged or altered gene function due to modification. As an alternative explanation to these genetic mutations epigenetic changes are being considered (Egger *et al.* 2004). Epigenetic changes are inherited through cell division and refer to alterations that affect the gene expression without changing the DNA sequence. It has been suggested that for instance DNA methylation may cause an abnormal silencing or activation of tumor suppressor genes and growth-stimulating genes, respectively (Feinberg and Tycko 2004). In several recent studies of HNPCC where no mutations were found in the MMR genes the subjects showed evidence of germline epigenetic modifications of both hMSH2 and hMLH1 (Chan *et al.* 2006, Hitchins *et al.* 2005, Suter *et al.* 2004). These epigenetic modifications are similar to inactivating mutations and generate a clinical phenotype reminiscent of HNPCC.

Identifying HNPCC-families

Because HNPCC is a hereditary disease it is important to find suitable patients and families for mutation analysis, both to verify occurrence of a specific mutation or, of equal importance,

to exclude a family from counseling and treatment. To be able to distinguish a sporadically emerged cancer, for instance caused by environmental factors, from a genetically inherited one and to classify this cancer as HNPCC, different criteria have been developed (Vasen *et al.* 1991). To date there are three different sets of criteria for HNPCC families. The Amsterdam criteria (AC) were the first to be stated, in 1991, by the International Collaborative Group of HNPCC (ICG-HNPCC), and were used as guidelines from 1993 (Vasen *et al.* 1991). But AC did not include guidelines applying to extracolonic cancer and also shown to be less adequate for discovering germline MMR mutations. To compensate for extracolonic cancer AC was extended in 1999 to the Amsterdam II criteria (ACII) (Vasen *et al.* 1999). The third criteria, the Bethesda criteria (BC), were created to include the aspect of microsatellite instability (MSI) that previously was lacking in both AC and AC II. BC were found to be less specific but more sensitive then the previous criteria (Umar *et al.* 2004). In a recent study it has been shown that BC will detect about 95% of Lynch syndrome related mutation carriers while AC II only will detect 42 % (Barnetson *et al.* 2006). All three criteria are described in more detail below.

Amsterdam criteria (AC):

1. Three relatives in the family are diagnosed with colorectal cancer. One of the affected is a first degree relative (sibling, parent or child) to the other two.
2. At minimum two following generations are affected.
3. At minimum one colon cancer is diagnosed earlier than 50 years of age.
4. Familial adenomatous polyposis (FAP) is excluded.

Amsterdam II criteria (AC II):

1. Three relatives in the family are diagnosed with colorectal cancer or a Lynch syndrome-associated cancer.
2. The other criteria are similar to those for AC.

Bethesda criteria (BC):

1. Family member(s) have two Lynch syndrome-associated cancers, including related extracolonic cancer, metachronous (two tumors continuously after each other) and synchronous (two tumors at the same time) colorectal cancers.
2. Family member(s) have colorectal cancer and a first degree relative with:
    - colorectal cancer diagnosed before 45 years of age.
    - and/or Lynch syndrome-associated extracolonic cancer diagnosed before 45 years of age.
    - and/or colorectal adenoma (benign glandule bulb) diagnosed before 40 years of age.
3. Family member(s) have an endometrial or colorectal cancer diagnosed before 45 years of age.
4. Family member(s) have a right-sided colorectal cancer that has an undifferentiated pattern.
5. Family member(s) have epilethial (cells at the surface of a tissue) colorectal cancer diagnosed before 45 years of age.
6. Family member(s) having adenomas diagnosed before 45 years of age.

Normally when a family fulfilling the Amsterdam criteria is discovered, the first degree relatives are immediately submitted to colonoscopy surveillance. Colonoscopy is initiated before the genetic testing because it may take a long time to get the results.

Analytic Methods

A cancer family clinic was established at the beginning of 1990 at the department of Clinical Genetics at Karolinska University Hospital. Here, individuals at risk are offered genetic counseling and regular colonoscopies. This study involves HNPCC families where at least the contact person (proband) for the family has been treated or diagnosed at the Karolinska University Hospital between 1987 and 2008.

To be able to verify or exclude mutations in individuals with a suspected HNPCC family history and to be able to offer genetic counseling, different analytic methods have been applied. For an overview of all techniques used at the Karolinska University Hospital in the Department of Clinical Genetics see figure 2.

When an individual comes in contact with the Karolinska University Hospital and after an initial counseling is judged to be an appropriate candidate (se the first part of figure 2) he/she is suggested to start the process of genetic testing. The genetic tests performed are described in more detail below (for a summary of all the steps see figure 2):

1. Microsatellite instability (MSI) test is used to detect any possible changes of length in the microsatellite sequences. When the test is positive it indicates a possible mutation. If the test is negative then the family history is used for empiric risk estimates.
2. The MSI-positive samples are then tested with immunohistochemistry (IHC) to see which of the proteins is not expressed. For instance if the MLH1 gene is mutated, IHC will show a negative result because the antibodies does not bind to the protein. The MMR genes that show a lack of expression are then sequenced to verify a mutation. In case all genes are expressed all three are sequenced.
3. If no point mutations are found in those genes that show a lack of expression in the IHC step, alternative screening methods to find insertions or deletions are applied.
4. If no mutation is found or if a mutation with unclear biological relevance is found, the BRAF gene is sequenced. This procedure is done because sporadic oncogenic mutations occur that are associated with MSI-positive tumors in the BRAF gene (Domingo *et al.* 2004). The existence of BRAF mutation excludes HNPCC.

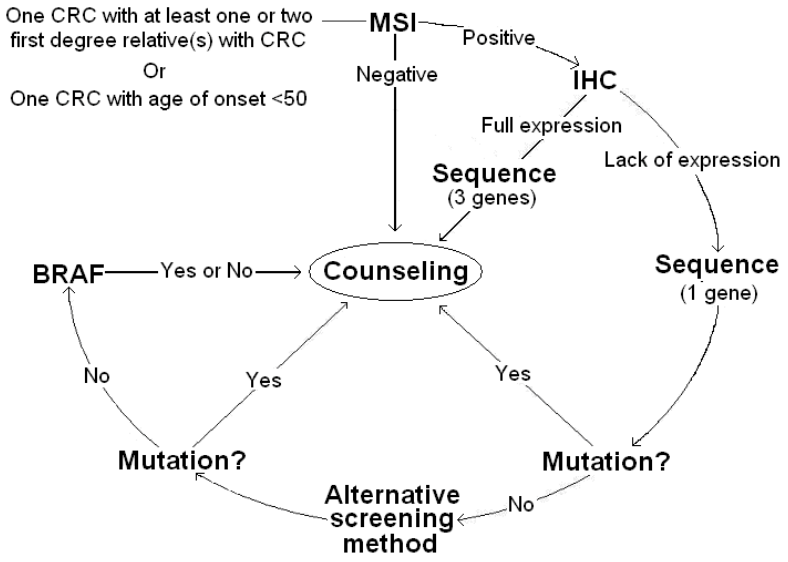

Figure 2. A schematic view over the diagnostic procedure used at the Karolinska University Hospital (modified from Lagerstedt Robinson *et al.* 2007). CRC = colorectal cancer; MSI = microsatellite instability; IHC = immunohistochemistry.

If an HNPCC related mutation is found, the individual is called to colonoscopy every second year and if no mutation is found every three to five years. It is important to consider empiric risks and keep in mind that finding a mutation in this case is as significant as excluding the possibility of one occurring.

Anticipation

Anticipation has been observed earlier in other genetic disorders such as Huntington's disease, fragile X syndrome and myotonic dystrophy. In 1992 it was established that extension of trinucleotide repeats was associated with in the disease locus in myotonic dystrophy (Mahadevan *et al.* 1992, Caskey *et al.* 1992). Since the discovery this genetic trend, has been acknowledged as the molecular basis of anticipation in myotonic dystrophy. Because of the MMR mutations it is highly interesting to see if a similar pattern can be observed in HNPCC.

In general, HNPCC-related cancers have an early age of onset (≈45 years) and the occurrence of anticipation, has been supported as well as rejected (Westphalen *et al.* 2005). Not many studies have been published in this field and the few attempts that can be found are divided in their answer. Some show clear evidence for anticipation (Westphalen *et al.* 2005) while others advocate the opposite that no anticipation occurs in HNPCC families (Tsai *et al.* 1997). All the studies dealing with this issue has approached the problem in a similar manner, by dividing individuals in birth cohorts and comparing the separate groups to see if a significant difference can be observed. For instance Tsai *et al.* (1997) found evidence against anticipation by dividing the study population into three cohorts depended on birth year; ≤1920, 1921-1930 and >1930. In a more recent study Westphalen *et al.* (2005) found evidence for genetic anticipation when dividing the patients into three different birth cohorts; born ≤1916, born 1916-1936 and born >1936. The approach was similar but the results differed.

Aims of the study

The aim of this study was to analyze anticipation in families with HNPCC, specifically:

1. To compare the presence of anticipation in the whole study population with different methods.
2. To compare the presence of anticipation in the whole study population with one consisting entirely of proven and obligate carriers (explained in more detail further down).
3. To determine the contribution to anticipation of the different HNPCC related genes (hMLH1, hMSH2 and hMSH6)

Results

Altogether 83 families with 298 individuals were included in this study. The individuals generated 358 generational differences that were analyzed by the cluster method and individual method. In the cluster method, mean age of onset of all individuals in one generation was subtracted from the mean age of onset of all individuals in the previous generation within the same family. In the individual method, every person in one generation was compared to every person in the previous generation within the same family. By using this two methods and additional statistical calculations, the occurrence of anticipation could be calculated (table 4). The two latter, but not the former, showed normal distribution, permitting use of a one sample t-test to calculate confidence intervals; for the former a Wilcoxon signed rank method was used instead. The results showed significant anticipation, with approximately 8 years earlier age of onset for each new generation whether all carriers were included, or just certain carriers (consisting of proven and obligate carriers). Certain carriers refer to individuals where the presence of a mutation is assured. The category "all" also includes individuals with a 50 % probability of carrying a mutation.

Table 4. Evidence of anticipation in different types of carriers.

Carriers	Statistical method	N	Median or Mean	95 % CI[5]	P-value[6]
All[1]	Wilcoxon Signed Rank	358	8.0[3]	7.0 to 9.0	<0.001
All[2]	One-sample T-test	358	7.96[4]	6.34 to 9.58	<0.001
Certain[2]	One-sample T-test	155	7.86[4]	5.55 to 10.18	<0.001

[1]Difference between generations calculated by the cluster method.
[2]Difference between generations calculated by the individual method.
[3]The median value.
[4]The mean value.
[5]Confidence interval.
[6]H_0=The CI includes 0 (no anticipation); H_1= The CI does not include 0 (anticipation occurs)

In order to see if all the genes related with HNPCC show evidence of anticipation by themselves or if one gene statistically compensates for the other, all patients with mutations in the same gene were tested by the individual method (table 5). Anticipation with 12 years earlier age of onset in forthcoming generations was shown in hMSH2 by analysis with the one-sample t-test. The differences in families with mutations in hMLH1 and hMSH6 were not normally distributed; the Wilcoxon signed rank method was applied. hMLH1 showed occurrence of anticipation with 7.5 years earlier development of cancer in the successive generation and hMSH6 5.5 years.

Table 5. Evidence of anticipation in different genes.

Gene	Statistical method	N	Median or Mean	95 % CI[3]	P-value[4]
hMLH1	Wilcoxon Signed Rank	144	7.50[1]	5.00 to 9.50	<0.001
hMSH2	One-sample T-test	124	12.27[2]	9.24 to15.29	<0.001
hMSH6	Wilcoxon Signed Rank	69	5.5[1]	2.50 to 8.00	<0.001

[1]The median value.
[2]The mean value.
[3]Confidence interval.
[4] H_0=The CI includes 0 (no anticipation); H_1= The CI does not include 0 (anticipation occurs)

Discussion

This study has provided significant evidence that anticipation occurs in 83 HNPCC families by calculating the difference between two generations. The children were found to have developed tumors 8 years before their parents (table 4). All certain carriers and the three MMR genes involved in HNPCC contributed to the occurrence of anticipation (table 4 and table 5). What is interesting to note is that the level of anticipation is much higher for hMSH2 than for the two other genes.

Comparison of results and methods

Unfortunately the results from previous publications cannot directly be compared with my results, since none of the earlier publications have approached this issue in same manner as I did. The basic approach adapted by the earlier studies, of course with some variation, was to divide the study population into different groups depended on birth age. For instance Nilbert *et al.* (2009) divided their patients in groups of children born ≤ 1930, ≤1935, ≤1940 and ≤1945 and then compared the parent-child pairs to find a significant difference. As they only took consideration of when the children were born, and then went back in the family history to find the parent, it was possible to obtain different groups with parent-child pairs. In this study four different birth depending groups were created, causing a fragmentation of the genetic family history lying beneath.

Even if the results are difficult to compare, the relative significance of the different methods can still be discussed. Anticipation is explained as earlier age of onset in successive generations and a generation is something relative within every family, all families are so to speak not on the same wavelength. Due to this, dividing families after birth years without taking into consideration the unique composition of generations within every family should influence the results negatively.

When dealing with a hereditary disease, such as HNPCC, it is impossible to know when the mutation first was introduced into the family. In calculations of anticipation the origin of the mutation is of great importance. Two individuals born in the same year from separate families might develop cancer at different ages, depending on how long the mutation has run in the family. Previous methods suggested do not show any consideration of this issue as they divide individuals in groups depending on birth year. This paper copes with the issue by embodying the uniqueness of *every family* within the mathematical methods.

Environmental bias

Although the methods used in this study are an improvement on the previous approach, there are still some environmental influences that cannot be considered. For instance, it has been mentioned earlier in the text that environmental causes like diet or physical activity can affect the onset of colorectal cancer. Another factor that certainly has an influence on anticipation is progress in diagnostic methods. As the methods become more and more efficient it is possible to detect a progressing tumor at an earlier age or stage than 50 years ago. Another bias is that all HNPCC families at the Karolinska University Hospital are in surveillance programs, meaning that patients undergo regular colonoscopies. This might also affect the results by earlier detection of tumors. Another bias is that the index case (proband) often contacts the hospital due to worries concerning earlier onset of cancer in the family. A way to correct this

would maybe be to exclude the last generation in every family, since surveillance programs first started in the late 80's, but unfortunately the data in this study were not large enough to do that.

The aim of this study was to analyze anticipation in HNPCC families, but the real purpose of the entire project is to improve the odds for survival and tumor prevention of patients by gathering as much knowledge as possible. Regardless of environmental issues, family mutations and technological progress all the results show a distinct pattern of anticipation. These results suggest that surveillance programs should be initiated at an earlier age but also that HNPCC can serve as a good candidate for unraveling genetic and/or epigenetic mechanisms determining age of onset in cancer.

Materials and Methods

Mutation and surveillance data have been collected from over 400 HNPCC patients, by going through a data base called 4D, patient journals, family pedigrees and other medical records. Information from a relatively large number of patients was collected (table 1), but only those who fulfilled certain criteria were included when calculating.

Table 1. Patients and families included in this study.

Variable	# of participants
Families	83
Individuals	298
Females	160
Males	138

Criteria for including and excluding patients

The following criteria were established to be able to distinguish between different kinds of carriers and to have guidelines to follow when studying the pedigrees:

1. A proven carrier is a person who has been tested by DNA analysis and found to have a specific HNPCC-related mutation.
2. An obligate carrier is a person known to have a mutation due to his/her position in the pedigree, in relation to relatives that are proven carriers.
3. A putative carrier is a first degree relative to a proven or obligate carrier; these individuals have a 50 % risk to carry the mutation.

Since anticipation depends on the age of onset, only patients with cancer were included in the study population. From the beginning however, everyone with an HNPCC related mutation according to journals and other medical records, was registered. Then by studying every family's pedigree and recording all proven carriers, other potential carriers (obligate and putative) could be extracted. For instance person 3.3 and 1.2 are both proven carriers of the same mutation and due to his position person 2.2 becomes an obligate carrier (figure 3). Since person 1.2 and 3.3 have the same mutation it is extremely unlikely that the mutation was not inherited through person 2.2. Similarly, person 2.7 becomes a putative carrier due to the position of persons 2.2, 1.2 and 2.5, who are first degree relatives carrying the mutation (figure 3). The total number of carriers after studying the pedigrees used in this paper is summarized in table 2.

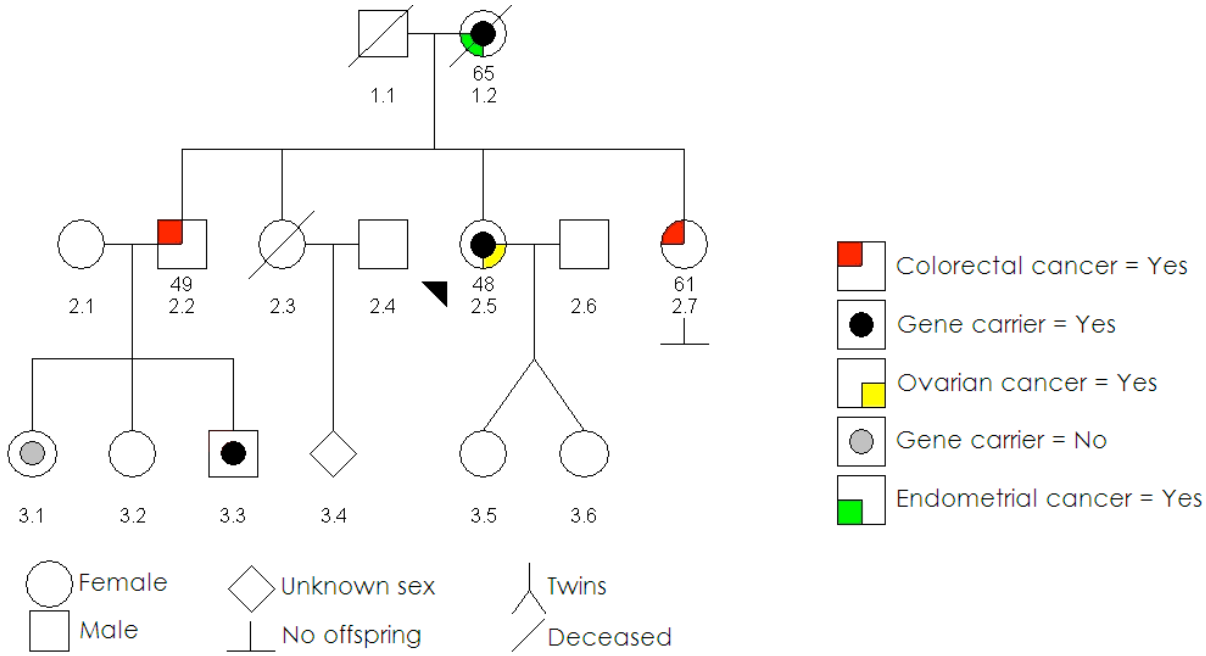

Figure 3. A pedigree of an HNPCC family. The number directly beneath every person with cancer shows the age of onset, the number a little further beneath every person represents their relative position in the pedigree and the black arrow shows the contact person (proband).

Table 2. Total number of carriers.

Carriers	**# of individuals**
Proven	161
Obligate	42
Putative	95

Because the data later were used to calculate anticipation depending on the mutation inherited within every family, all carriers (proven, obligate and putative) were also divided into groups by genes (table 3).

Table 3. Total number of individuals with mutation in a certain gene.

Gene	**# of individuals**
hMLH1	143
hMSH2	107
hMSH6	46

Mathematical methods

The differences in age of onset between the previous and the next generation ("generation 1" – "generation 2") were calculated to create a "data base" for statistical analysis with two different methods.

In the cluster method, the mean age of onset in every generation in every family was calculated. Then the mean age of onset in the next generation was subtracted from the mean age of onset in the previous generation *within the same family*. This means that for every two generations one difference could be obtained. For instance a hypothetical family, Family 1, with four individuals, two in generation 1 and two in generation 2, would have the same statistical leverage as Family 2 with eight members, three in generation one and five in generation two. To compensate for this, the differences between generations was then used

the same number of times as the *actual number* of differences between generations. For instance, if the mean difference between generation one and generation two in Family 1 was 5.50, this value would then be used four times (2x2). If the mean difference between generations in Family 2 was 6.67 then it would be used 15 times (3x5).

In the individual method every patient's age of onset in one generation was individually subtracted from the age of onset of every patient in the previous generation, *within the same family*. This means that in Family 1 one would obtain four separate differences and in Family 2, 15 separate differences. With this procedure all the patients had the same statistical leverage, no compensation is needed.

As observed in Family 2 it is important to note that the number of differences will not always be equal to the number of individuals.

Statistical methods

At first a Kolmogorov-Smirnov test was used to prove a normal distribution (Gaussian distribution). If a normal distribution could be proven then a one-sample T-test was used to create a confidence interval (CI) of the mean value. If a normal distribution wasn't obtained a nonparametric test called Wilcoxon Signed Rank was used to create a CI of the median value. Anticipation is proven to occur if the confidence interval does not include zero. A significance level of ≤ 0.05 (5%) was considered sufficient. MiniTab, version 15.1.20.0, was used to do all statistical calculation.

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