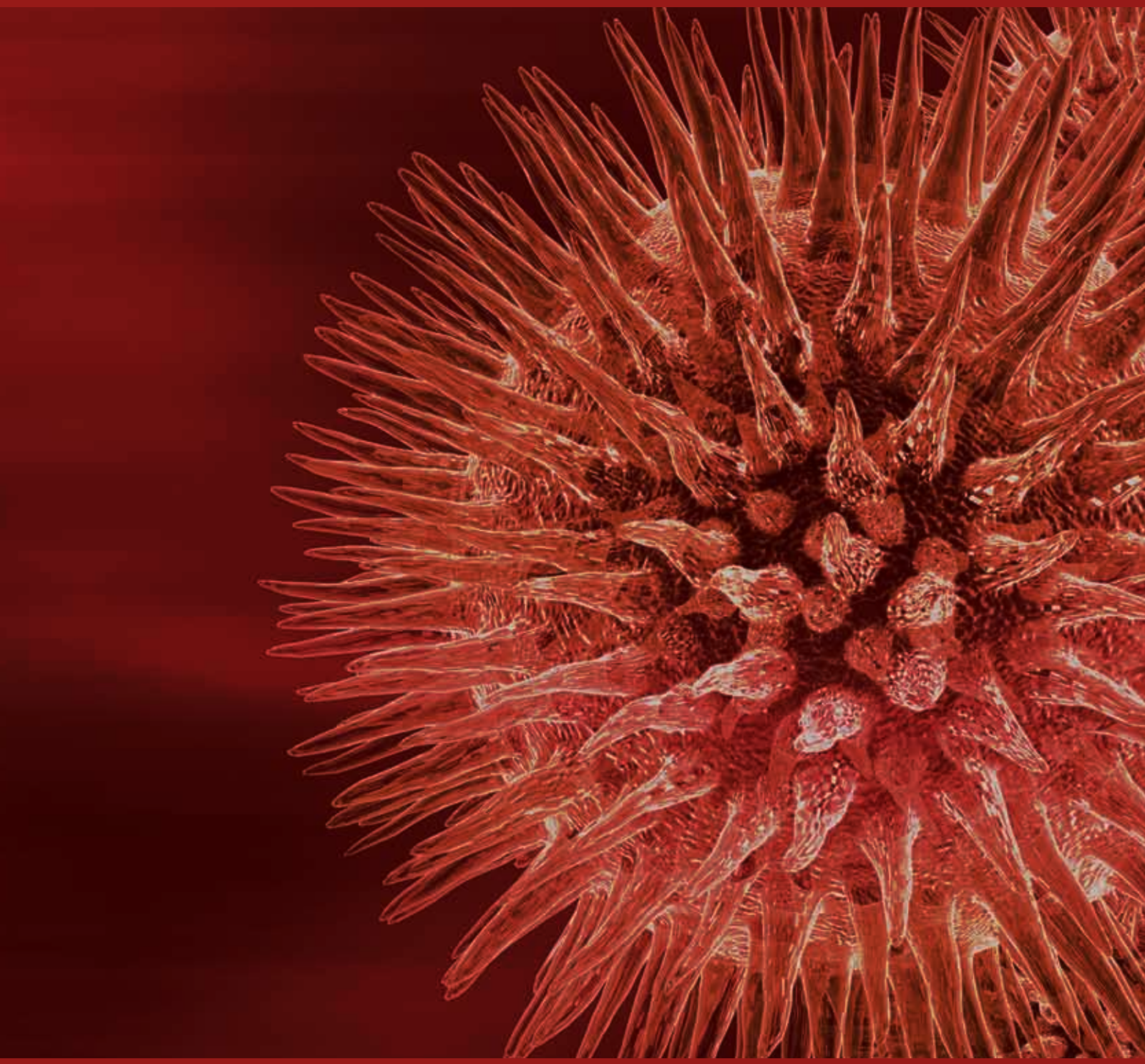
BioMed Research International

Hereditary Tumours

Guest Editors: Francesco Baudi, Kathleen Claes, Anna Di Gregorio, Serena Masciari, and Florentia Fostira



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Editorial
Hereditary Tumours

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Hereditary tumours account for 3–10% of all cancers. Greater susceptibility of the subjects to these types of malignancies is thought to be the result of genetic mutations in highly penetrant genes transmitted as autosomal dominant traits. Mutations in such susceptibility genes result in familial predisposition to developing a characteristic spectrum of diseases (collectively known as Hereditary Cancer Syndromes). While these mutations do not cause the disease *per se*, their carriers exhibit a higher risk (usually ≥50%) of developing the disease during their lifetime.

Most of the studies carried out in this field have focused on the most common hereditary tumours, breast cancer and colorectal cancer, for which the major susceptibility genes, BRCA1 and BRCA2 and MLH1 and MSH2, respectively, were identified about 20 years ago. The discovery of these genes has made it possible to translate, in a relatively short amount of time, basic research findings into clinical practices (bench to bedside) by contributing appropriate diagnostic strategies. These include genetic tests that are currently used to screen families for the identification of mutation carriers and to improve both prevention and management of hereditary cancers. At the same time, the study of cancer-specific signaling pathways and pathogenetic mechanisms has allowed the development of targeted therapies.

In this special issue, P. Apostolou and F. Fostira provide an overview on the current knowledge of the genes causing predisposition to breast cancer. Besides BRCA1 and BRCA2, the most frequently mutated genes, the authors also review the contribution of additional genes to hereditary breast cancer syndromes that have recently emerged through whole-genome analyses. Mutations in these newly discovered genes account for the heterogeneity found among different syndromes and result in differential diagnoses.

About one hundred syndromes characterized by Mendelian inheritance can be ascribed to the presence of very rare alleles [1], yet these rare alleles are found in only 20–40% of cancers where inheritance is suspected. Given this paradox, other mechanisms must be at play, such as the presence of specific genomic structural variants that might escape common methods of detection based on Sanger sequencing. In their research article, F. Duraturo et al. show the application of multiplex ligation-dependent probe amplification (MLPA) for determining the contribution of large genomic rearrangements of MLH1 and MSH2 genes in a population of Lynch syndrome patients where analysis for point mutations was previously negative. The MLPA allows to retrieve an even small percentage of "missing heritability" due to structural variants other than classic substitutions or small indels. We can expect that, when the actual contribution of these structural variants to various diseases is elucidated, we will be able to perform advanced genetic testing, including a more comprehensive evaluation of additional structural variants, such as large-scale copy number variation (CNV) or epimutations.

The deterministic model, which attributes an inherited susceptibility to mutations in high-penetrance cancer-specific genes, has been joined more recently by an alternative "polygenic" model that is based on the cumulative role of multiple low-penetrance alleles [2]. Many of these loci have been identified by genome-wide association (GWA) studies

and a list of those involved in susceptibility to breast cancer is reviewed by P. Apostolou and F. Fostira. An estimation of the penetrance of these rare alleles and their corresponding risk is complicated; in turn, this makes genetic counselling very difficult especially in cases where prevention strategies are based on prophylactic surgery. The advent of novel sequencing technologies, from whole-genome to exome-sequencing, will likely accelerate progress in this field.

Since cancer is characterized by a peculiar gene expression profile, another mechanism that could explain "missed heritability" is posttranscriptional regulation. The discovery of microRNAs (miRNAs), small noncoding single-stranded RNA molecules that act as master switches in the regulation of gene expression, has expanded the research on cancer susceptibility to these RNAs. miRNA-mediated silencing may be shaping the characteristic gene expression signatures of different cancers and defining tumour subtypes. R. Iuliano et al. summarize the main effect on tumour susceptibility of single-nucleotide polymorphisms (SNPs) included in regions related to miRNA-dependent pathways. SNPs are categorized into three main divisions: SNPs that are mapped within miRNA genomic regions and may participate in carcinogenesis by altering the expression of tumour-related microRNAs, SNP variants located in $3'$-UTRs of miRNA-targeted genes that can influence tumour susceptibility by destroying or creating miRNA binding sites, and SNPs in genes encoding microRNA-processing complexes that influence the expression of proteins involved in the miRNA biogenesis pathway.

The expansion of the catalog of variants and the redefinition of risk factors that will ensue should be integrated with a deeper knowledge of the consequences on the pathways that these genes control in order to design new, more efficient strategies for prevention and therapy. A useful contribution to the understanding of the mechanisms of tumour susceptibility may also come from the study of rare cancers, as well demonstrated by the review of Y. Nibu et al. Here, the authors describe the molecular mechanisms underlying notochord formation and the onset of chordoma, a rare bone cancer for which sporadic as well as hereditary forms are known. The review is focused on the *Brachyury* gene, which is the main regulator of the notochord formation, a causative agent of chordoma and a potential therapeutic target. The authors describe a number of potential mechanisms of action of this gene during tumourigenesis, ranging from modulation of a series of genes downstream of this transcription factor, the dosage-dependent effects on them, the interaction of *Brachyury* with other proteins that may affect its function, up to molecules and signaling pathways that regulate its expression. The implication of a developmental gene in the pathogenesis of the disease supports the cancer stem cell hypothesis that may open the search for potential therapies targeting *Brachyury*.

In cancers showing a familial component without known inheritable causes and satisfying disease management, the implementation of appropriate preventive measures at an early age for people considered to be at higher risk is mandatory. Particular attention should be given to subjects with family history of cancer without tractable mutations in major susceptibility genes. These issues are well explained in the manuscript of G. Corso which addresses the issue of familial intestinal gastric cancer (FIGC). Since the genetic basis of susceptibility to this cancer is largely unknown, careful protocols for monitoring and management of asymptomatic patients have to be developed. The proposed management flowchart follows the presentation of a very original clinical case centered on the family history of gastric cancer of the Roncalli family, the family of Pope John XXIII, the Pope of the Catholic Church who died in 1963 of peritonitis resulting from perforation of a gastric cancer lesion.

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Francesco Baudi

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Review Article

From Notochord Formation to Hereditary Chordoma: The Many Roles of *Brachyury*

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Chordoma is a rare, but often malignant, bone cancer that preferentially affects the axial skeleton and the skull base. These tumors are both sporadic and hereditary and appear to occur more frequently after the fourth decade of life; however, modern technologies have increased the detection of pediatric chordomas. Chordomas originate from remnants of the notochord, the main embryonic axial structure that precedes the backbone, and share with notochord cells both histological features and the expression of characteristic genes. One such gene is *Brachyury*, which encodes for a sequence-specific transcription factor. Known for decades as a main regulator of notochord formation, *Brachyury* has recently gained interest as a biomarker and causative agent of chordoma, and therefore as a promising therapeutic target. Here, we review the main characteristics of chordoma, the molecular markers, and the clinical approaches currently available for the early detection and possible treatment of this cancer. In particular, we report on the current knowledge of the role of *Brachyury* and of its possible mechanisms of action in both notochord formation and chordoma etiogenesis.

1. Chordoma: Epidemiology, Classification, and Histopathological Characteristics

Chordomas are very rare tumors that affect roughly one in a million individuals; the incidence in the US is ~300 new cases per year [1]. Yet, this rare neoplasm represents up to 4% of primary malignant bone tumors [2] and 20% of primary spine tumors [3].

Chordomas are classified on the basis of their location along the spine and their histological type. Depending on their location, chordomas are predominantly subdivided into clival (or skull-base), sacrococcygeal, cervical, thoracic, and lumbar. Even though historically the sacrococcygeal region was regarded as the most frequently occurring site for the formation of these tumors (e.g., [2]), recent studies have shown their almost equal distribution in the skull base, mobile spine, and sacrum [1].

Chordomas rarely occur in people below 40 years old; however, numerous cases of pediatric chordomas have been reported (e.g., [4, 5]) and generally were associated with cranial locations [6]. While cranially located chordomas affect both genders equally, sacrococcygeal tumors are more frequent in males, with the male : female ratio being approximately 2 : 1 ([7], and references therein). African-American individuals have been reported to be less frequently affected by chordoma [1], while Hispanic patients were found to have a significantly higher survival rate [6].

In addition to the more frequent axial tumors, extra-axial chordomas have also been reported. The location of extra-axial chordomas ranges from wrist [8] to feet [9]. These tumors have traditionally been identified through immunohistochemical studies [10, 11], and, more recently, through the use of the *Brachyury* gene as a novel diagnostic marker that distinguishes chordomas from similar lesions, such as myoepitheliomas and chondrosarcomas [9, 12].

Histologically, chordomas are categorized as classical (or conventional), chondroid, and dedifferentiated (e.g., [3]). The first microscopic characterization of chordomas dates back to 1857, when Virchow first identified the cells typical of this tumor and described them as "physaliferous" (Greek for "bubble-bearing") because of the foamy appearance

of their cytoplasm that contains multiple vacuoles [13]. Ultrastructural studies have indicated that the vacuoles can be divided into two subtypes, smooth-walled and villous, based upon the absence or presence of microvilli, respectively [14]. Physaliferous cells are typical of classical chordomas, appearing as groups of gray-white large cells separated by fibrous septa into lobules and surrounded by a basophilic extracellular matrix rich in mucin and glycogen [7, 15]. This is the most frequent type of chordoma. Its distinctive histological appearance led Müller to hypothesize, in 1858, that these tumors were of notochordal origin [16]; later, in 1894, Ribbert first introduced the term "ecchordosis physaliphora" [17], which is currently used to designate hamartomatous lesions of notochordal origin. Notochordal hamartomas are considered the benign counterparts of chordomas and are usually asymptomatic [18, 19]. While both ecchordosis physaliphora and chordoma are composed mainly of physaliphorous cells, stain for vimentin, the S-100 protein, epithelial membrane antigen, and low molecular weight cytokeratins, and are both negative for high molecular weight keratins [20], it is still unclear whether ecchordosis physaliphora can be a precursor of chordoma [19]; further investigations are needed to address this open question.

Chondroid chordomas show histological features resembling both chordoma and chondrosarcoma, a malignant tumor of the bone and soft tissue (e.g., [21]). This histological variant accounts for 5%–15% of all chordomas and up to 33% of all cranial chordomas, being preferentially found on the spheno-occipital side of the skull base [3]. Despite an appearance that resembles hyaline cartilage, these tumors retain an epithelial phenotype and express specific chordoma markers, including cytokeratin and S-100, which are not found in cartilaginous tissue; this has suggested their alternative, more appropriate classification as "hyalinized chordomas" [22]. Dedifferentiated chordomas are also rare, <10% of chordomas, and are characterized by sarcomatous regions, which are comprised of spindle-shaped polygonal cells (e.g., [23]). An important connection has been observed between the histological category of a chordoma and its ability to metastasize [24]: chondroid chordomas are the least aggressive, while dedifferentiated chordomas are the fastest-growing, more metastatic variety [3].

2. Therapeutic Approaches: Past and Present

Since the late 1970s, treatment of chordoma has traditionally relied on *en bloc* surgical removal [25]; this technique has been very effective for sacral chordomas, although its applicability is obviously limited in the case of clival chordomas. Radical resection of the tumors and their capsules has proven to be considerably more effective than subtotal excision because it reduces the extent of cell seeding and, consequently, the risk of recurrence [26–28]. Advanced imaging techniques, including CT and MRI scans, allow for early detection of small intraosseous tumors and their complete resection [29, 30]. Since chordomas are radioresistant tumors, radiation therapy is usually employed in combination with surgery and mainly relies upon high-dose proton beam [31] and, to a more limited extent, on either helium- or carbon-ion therapy [32, 33].

Unfortunately, as slow-growing tumors, chordomas are resistant to conventional chemotherapies [34], and the use of alkylating agents, alkaloids, and related treatments has been reported to be modestly effective mostly on dedifferentiated chordomas [35]. However, increasing knowledge of the genetic markers of chordomas has led recently to the use of molecularly targeted therapies. The finding that chordomas display overexpression of constitutively active phosphorylated forms of platelet-derived growth factor receptor-(PDGFR)-b and of KIT receptors [36] prompted the use in chordoma patients of imatinib, a tyrosine-kinase inhibitor (TKI) drug with specificity for the kinase domains of these receptors. Imatinib was used with some success in clinical trials [37] alone or in combination with the immunosuppressant sirolimus (a.k.a. rapamycin) [38]. Another TKI, sunitinib, which targets multiple receptor tyrosine kinases and is used mainly for the treatment of gastrointestinal stromal tumors, has shown therapeutic efficacy against chordomas (e.g., [39]). It seems likely that the effects of TKI drugs may extend to other phosphorylated receptors found to be altered in chordomas, such as PDGFR-a [40], and to kinases that participate in additional pathways, such as Akt and mTOR, whose targets, TSC2 and the translation initiation factor binding protein EIF4EBP1, are found in a phosphorylated state in chordoma cells [41]. Other signaling pathways distinctive of chordomas that have been targeted are those linked to the epidermal growth factor receptor (EGFR); inhibitor drugs, such as cetuximab, gefitinib, and erlotinib, have been used in a small number of patients [42, 43]. The use of 9-nitrocamptothecin (9-NC), an inhibitor of topoisomerase I, was also limited to a small group of patients and seemed to delay cancer progression [44].

The development of chordoma cell lines in the past decade has also greatly widened the knowledge of the chordoma-specific molecular profile and has accelerated the identification of novel therapeutic targets. The first human chordoma cell line, U-CH1, was developed in 2001 [45] and has been used recently to test the efficiency of the various treatments described above [46]. Following the establishment of U-CH1, other cell lines were developed, including CH22 [47] and a cell line derived from a rare extra-axial chordoma, originally located in the scapula [48]. Other chordoma cell lines, CH 8 and GP 60, have also been generated successfully and tested for their ability to grow in different media [49]. Studies on these cell lines have identified new potential therapeutic agents, such as inhibitors of the transcription factor STAT3 [49]. In addition, work in chordoma cell lines, and parallel studies of familial chordomas, have revealed the role of another transcription factor, Brachyury, in the formation of this tumor. Short-hairpin RNA (shRNA)-mediated silencing of *Brachyury* in the cell line JHC7, derived from primary sacral chordoma, has shown that the downregulation of this transcriptional regulator leads to growth arrest [50]. Chromosomal gain at the *Brachyury* locus, seen in the MUG-Chor1 cell line, is found in a subset of familial chordomas [51] and is described in detail below.

3. Genetic and Cytogenetic Hallmarks of Chordoma

As described above, chordomas were classically identified on the basis of their histological features and their immunoreactivity for S-100 and markers such as epithelial membrane antigen (MUC1) and cytokeratins. However, the immunoreactivity for S-100, which is shared among chordomas and chondrosarcomas, made the assignment of tumors to either category quite challenging. This was particularly true when only small biopsies could be obtained, as in the case of clival chordomas (e.g., [52]). Another genetic marker of chordomas is chondroitin sulfate proteoglycan 4 (CSPG4), which has also been targeted for immunotherapy; however, this antigen is expressed only by a fraction (~62%) of these tumors [53] and could therefore produce false negative diagnoses. These limitations prompted a widening of the search for distinctive chordoma markers to transcription factors. The Sry-type HMG-box transcription factor Sox9, which is required for the maintenance of notochord integrity [54], was found to be expressed in both kinds of tumors [55]. Conversely, the detection of Brachyury, a member of the T-box family, used in combination with cytokeratin, provided a considerably accurate distinction of chordomas from chondrosarcomas [55]. Further studies revealed specific expression of *Brachyury* in >90% of the chordomas analyzed, and validated its use as a specific marker of this tumor [52, 53, 56]. Nevertheless, it is noteworthy that *Brachyury* is overexpressed in a number of tumor types, including lung cancer, and is a potent mediator of epithelial-mesenchymal transition (EMT) [57, 58], one of the main mechanisms responsible for metastasis (e.g., [59]).

Numerous cytogenetic abnormalities have been reported in chordomas and have been used to aid in their classification, the most common being the partial or complete loss of chromosome arms 1p and 3p, and the partial or complete gain of chromosomes 7 and 20 [60]. In particular, chromosomal regions 1p36 (the *RIZ* locus, encoding a zinc-finger protein; [61]), 1q25 (encompassing the hereditary human prostate cancer susceptibility locus *HPC1*, [62]), 2p13 (the *TGF-alpha* locus), and 7q33 (the *AKR1B10* locus, encoding an aldo-keto reductase; [63]) display either deletions or amplifications in primary chordomas [64]. Other frequent deletions reported in chordoma occur in the *CDKN2A* and *CDKN2B* loci, both on chromosome 9p21, which encode the cyclin-dependent kinase inhibitors known as p16 and p15, respectively [65]. Of note, chordomas are among the cancers that display a newly described mechanism of chromosomal disruption, called chromothripsis, which is found in ~25% of bone cancers and involves the massive fragmentation of one or more chromosomes, followed by their imperfect reassembly [66].

4. Duplications and Mutations of *Brachyury* in Hereditary and Sporadic Chordomas

Some of the most relevant advancements in the search for the molecular bases of chordoma are the result of systematic studies of families affected with this cancer. The first of these analyses showed genetic linkage to chromosome 7q33 [63], which was corroborated by later research [67]; however, an additional study on the same group of families revealed that one of the individuals affected by chordoma had not inherited the 7q33 haplotype, thus prompting the search for additional susceptibility loci [68]. In turn, these efforts led to the identification of a 6q haplotype shared by all affected individuals in one of the families and defined a minimal disease region spanning ~5 Mb [68]. Through the analysis of copy number variants (CNVs), this interval was found to contain regions of 6q27 duplicated to various extents. Eventually, the duplicated areas shared by all families were narrowed to an ~100 kb interval that contains only one locus, *Brachyury* [68, 69].

Subsequent work analyzed the frequency of *Brachyury* amplifications in 181 sporadic chordoma samples: 7% of the tumors displayed amplification of the *Brachyury* locus, 39% of the tumors were polysomic for chromosome 6, and 4.5% of primary tumors showed a minor allelic gain of *Brachyury* [70]. No relationship was observed between the frequency of amplifications in these sporadic chordomas and the region of origin of the tumors [70]. Of note, no germline alteration of the *Brachyury* locus was identified in non-neoplastic tissue from 40 patients, indicating that the copy number gain is somatic [70].

In sum, these studies demonstrated that chromosomal aberrations resulting in copy number gain of *Brachyury* are found in both sporadic [70] and hereditary chordomas [68]. Consistently, the expression of this gene is critical for the proliferation of chordoma cell lines *in vitro*, as shown by the results of the silencing experiments reported above [50]. Furthermore, the combination of traditional Sanger sequencing with recently developed whole-exome sequencing methodologies allowed an extensive genotyping study of germline DNA from 45 individuals with sporadic chordoma, focused on the *Brachyury* coding region [71]. The results revealed a recurring single nucleotide polymorphism (SNP), rs2305089 that lies in exon 4, which encodes part of the DNA-binding domain of Brachyury [71]. This mutation, therefore, alters the DNA-binding properties of this transcription factor, suggesting that the misregulation of some of the genes controlled by Brachyury could be a mechanism underlying the genesis of chordoma. Brachyury-downstream genes identified so far in various model organisms are discussed in detail in a later section.

5. The Role of *Brachyury* in Notochord Evolution and Development

Long before its identification as a marker and a possible cause of chordoma, *Brachyury* was already well known as a major regulator of notochord formation. The notochord is the main defining feature of the chordate phylum, which includes humans and all other vertebrates. This embryonic structure provides crucial support to the developing embryo, patterns the central nervous system, and ensures the proper development of a variety of organs, from heart and aorta to liver and pancreas [72]. As the ossification of the spine

proceeds and vertebral bodies form, the notochord regresses and eventually its remnants become the *nuclei pulposi* (NP) of the intervertebral discs. The histological identity of these cells remained controversial, since cells of the NP were described as either "chordoid," that is, notochordal, or as "chondroid," that is, as chondrocytes [73]. It has recently been clarified, through lineage-tracing experiments, that notochord cells are the embryonic precursors to all NP cells and to the mature intervertebral discs [74].

The *Brachyury* (Greek for "short tail") mutation was first identified in 1927 by Nadine Dobrovolskaïa-Zavadskaïa through the observation of short-tailed mice, which were later found to be heterozygous for a mutation in the eponymous locus. Subsequent studies confirmed that the mutation was dominant, attributable to a single factor, and accompanied by abnormalities of the posterior skeleton [75], particularly in the number of presacral vertebrae [76]. It was also determined that homozygous *Brachyury* embryos die *in utero* by gestation day 11 due to severe defects in the formation of the allantois [77]. Interestingly, these embryos fail to form a notochord and, as a consequence, show severe abnormalities in the development of the neural tube and somites [78].

The identification of the gene *Brachyury* (or "*T*," for "tail"), which in mouse is part of the *t*-complex that spans 40 cM on chromosome 17 [79], did not occur until 1990; the gene was isolated by positional cloning [80] and found to be expressed in notochord and primitive-streak mesodermal cells [81]. Three years later, the Brachyury protein was classified as a novel sequence-specific transcription factor [82]. Parallel studies identified *Brachyury* orthologs in *Xenopus*, zebrafish and chick [83–86]. Of note, the immature NP cells of the intervertebral discs were found to express both Brachyury mRNA and protein [56, 87].

The discovery of *Brachyury* orthologs in basal chordates, such as the ascidians *Halocynthia* and *Ciona* [88, 89] and the amphioxus *Branchiostoma* [90], and the remarkable conservation of their function underscored the paramount role played by this developmental regulator in the appearance of the notochord, the main event in the evolutionary history of the phylum Chordata. However, the appearance of *Brachyury* genes predates that of the notochord by several million years. In fact, although *Brachyury* orthologs have not been found in plants, genes related to *Brachyury* have been identified recently in the fungus *Spizellomyces punctatus* and in the amoeba *Capsaspora owczarzaki* [91], shifting the long-standing paradigm that considered *Brachyury* and its related genes as metazoan-specific innovations. Rather, it has become evident that the expansion of the repertoire of genes encoding transcription factors related to Brachyury accompanied the evolution of multicellularity in the animal kingdom. Indeed, Brachyury is the founding member of a family of transcription factors that share related DNA-binding domains and are therefore collectively designated as T-box (Tbx) proteins [92]. The availability of full genomic sequences for numerous animal species has confirmed the nearly ubiquitous representation of *Brachyury* and other *Tbx* genes throughout different phyla. For example, single-copy *Brachyury* and *Tbx2/3* orthologs have been reported in the placozoan *Trichoplax adhaerens* [93] and *Brachyury* and other *Tbx* genes have been described in sponges (e.g., [94]). Likewise, *Brachyury* and *Tbx2/3* orthologs have been reported in *Pleurobrachia pileus*, a member of Ctenophora (comb jellies), one of the first metazoan phyla [95].

Placozoan *Brachyury* is expressed in scattered individual cells that do not coincide with known anatomical structures [93], and its function in this simple organism is still unknown. However, several lines of evidence suggest that the ancestral function of Brachyury was the regulation of morphogenetic movements [96]. Consistently, in the sponge *Suberites domuncula*, *Brachyury* is expressed at the time when cell-cell and cell-matrix interactions are being established, suggesting that it might regulate cell adhesion and migration. The ctenophore *Mnemiopsis leidyi* expressed *Brachyury* (*MlBra*) in ectodermal cells around the site of gastrulation as well as in cells deriving from the blastopore; morpholino oligonucleotide-mediated knockdown supports its involvement in gastrulation movements [97]. In the sea anemone *Nematostella vectensis*, a basal cnidarian, expression of *Brachyury* is restricted to a circle of cells surrounding the blastopore [98].

In protostomes, a large division of animals including arthropods, mollusks, annelids, and other less-known taxa, the blastopore is the embryonic region that gives rise to the mouth. *Drosophila Brachyury,* called *brachyenteron*, is required for the formation of the hindgut and of the midgut constrictions, and for the elongation of Malpighian tubules [99]. This function in the formation of posterior structures is conserved in other arthropods, including the beetle *Tribolium* and the grasshopper *Locusta* [100]. Notably, Brachyenteron synergizes with the winged-helix transcription factor Forkhead to specify the caudal visceral mesoderm of *Drosophila*, a remarkable parallelism with the roles played in mesoderm formation by chordate Brachyury and Forkhead (Fox) orthologs [101, 102].

Lastly, *Brachyury* orthologs have also been studied in the two main phyla of non-chordate deuterostome animals, echinoderms (e.g., sea urchins and sea stars) and hemichordates (e.g., acorn worms). In the sea urchin *Lytechinus variegatus*, microinjection of a dominant-negative form of its *Brachyury* ortholog caused a block in gastrulation [103]. While there are as yet no functional studies of *Brachyury* in hemichordates, it has been shown that in the acorn worm *Ptychodera flava*, *Brachyury* is expressed in the blastopore at the time of gastrulation, and later in the stomodeum, the precursor of the mouth and anterior pituitary gland [104].

Together, these functional analyses in different representative organisms along the phylogenetic tree indicate that the ancestral function of *Brachyury* was the promotion of cell movement and adhesion, which are fundamental for both morphogenesis and tumorigenesis. As the evolution of multicellular organisms proceeded, this gene became involved in the specification of an area of the blastopore with distinctive properties in axis formation, and later on turned into a driver of mesoderm specification (e.g., [105]). Eventually, possibly through the synergistic interaction with other transcription factors, such as members of the Fox family, Brachyury acquired its crucial role in notochord formation.

6. Possible Mechanisms of Action of Brachyury during Tumorigenesis: The Brachyury-Downstream Gene Battery

Brachyury has been reported to act as a transcriptional activator, although it possesses two repression domains in its C-terminal region [106]. Crystallographic studies elucidated the conformation of the DNA-binding domain (T-domain) of *Xenopus* Brachyury (Xbra) bound to a 24-nucleotide palindrome and revealed the peculiar interactions between the C-terminus of the Xbra protein and the minor groove of DNA [107]. Subsequent studies using electrophoretic mobility shift assays (EMSA) have shown that Xbra and other Brachyury orthologs can also bind sequences that correspond to roughly half of the palindrome originally identified by Kispert and Herrmann [82]. In fact, numerous functional non-palindromic binding sites, also designated as "half-sites," with the minimal generic consensus sequence TNNCAC [108], have been identified in different model systems (e.g., [109–111]). A longer, non-palindromic consensus binding site, RWWNTNRCACYT, was also identified for *Drosophila* Brachyenteron based on its interaction with the *orthopedia* gene [112]. The *orthopedia* regulatory region contains multiple copies of this site, which are bound cooperatively by Brachyenteron and are also recognized *in vitro* by mouse and *Xenopus* Brachyury [112].

As the knowledge of Brachyury binding preferences continued to accumulate, the quest to identify its downstream genes gained momentum and led to the discovery of numerous Brachyury targets in chordates, but also in nonchordate model systems, such as the sea urchin [113]. In chordates, Brachyury targets were first identified in *Xenopus* and the invertebrate chordate *Ciona intestinalis* and, more recently, in zebrafish, mouse stem cells, and chordoma.

Studies in *Xenopus* led to the identification of five putative targets of Xbra: the signaling molecule Xwnt11, the zinc-finger transcription factor Xegr-1, a member of the BTG/Tob family of antiproliferative proteins, Xbtg1, a protein of unknown function, BIG3/1A11, and the homeodomain transcription factor Bix1 [114, 115]. Follow-up studies on Xwnt11 indicated that Xbra controls gastrulation movements through this signaling molecule [116]. Additional work revealed that Xbra binds the regulatory region of *eFGF* to regulate its expression [109, 117]. Together, these results began to delineate the gene regulatory network controlled by Brachyury, first during gastrulation and later in mesoderm formation.

The identity of numerous notochord-specific targets of Brachyury was later revealed by studies in *Ciona intestinalis*, since in this and other tunicates the expression of *Brachyury* (*Ci-Bra*) is confined to the notochord [89]. A subtractive screen aimed at identifying Ci-Bra-downstream genes first uncovered ~40 genes expressed in notochord cells [110, 118–121]. Interestingly, in contrast to the earlier results in *Xenopus*, most of the validated notochord Ci-Bra targets encode various structural proteins, including a non-muscle tropomyosin, fibrillar collagen, laminins, and thrombospondin [110, 122, 123]; however, components of the Wnt/planar cell polarity pathway, such as Prickle, and mediators of TGF-beta and NF-kappaB signaling were also discovered [121]. Subsequent studies expanded the number of possible components of the Ci-Bra-downstream regulatory hierarchy [119, 122] and recent genome-wide chromatin occupancy studies (ChIP-chip assays) suggested that the number of loci directly bound by Ci-Bra in early *Ciona* embryos is close to 2,000 [124]. In addition, notochord transcription factors of the STAT, Fos, AFF, Krüppel-like (Klf), and NFAT5 families were recently indicated as Ci-Bra targets, suggesting that the gene regulatory network controlling formation of the notochord in this simple chordate is multitiered, and that these transcription factors might have been reiteratively recruited for notochord development by other chordates [125]. Of note, in vertebrates *NFAT5* was found to be expressed in the NP, where it is required for the survival of the notochord cells in response to hyperosmotic stress [126].

In zebrafish, a *Brachyury* ortholog, no tail (ntl), has been found to control the expression of numerous target genes, including transcription factors as well as genes that participate in signaling and differentiation pathways [127]. These genes are involved in a variety of cellular processes, including morphogenetic movements (e.g., *wnt11*, *snail1a*, *connexin 43.3,* and *tbx16*), muscle specification (e.g., *mesogenin1* and *myoD*), and generation of posterior identity (e.g., *tbx6*, *vent*, *vox*, *fgfr4,* and *cdx* genes) [127]. Among the Ntl target genes is also *floating head*, the ortholog of the mammalian homeobox transcription factor gene *Noto*, which is required for the formation of the posterior notochord [128].

Putative target genes of mouse Brachyury, 396 in total, have been recently identified in mouse differentiating stem cells [129]. Similarly to zebrafish Ntl, mouse Brachyury controls several transcription factors of the homeobox, winged-helix, paired box, zinc-finger, and odd-paired families; it also regulates components of the Wnt signaling pathway, the growth factor Fgf8, cytoplasmic dyneins, and orthologs of the transcriptional repressor Snail [129]. However, this study also identified Brachyury targets that were not identified by the zebrafish study: Axin2, a negative regulator of the Wnt pathway, gamma-catenin/Jup, Fgf8, and Wnt3A; interestingly, ChIP-qPCR experiments showed that these genes were also bound by human Brachyury in hESC-derived mesoderm cells [129].

It is noteworthy that one of the recurring targets of Brachyury in different animals, including *Ciona*, zebrafish and mouse, is the gene *Snail* [124, 127, 129]. *Snail* encodes a transcriptional repressor that, along with transcription factors of the ZEB, Slug, and Twist families, is a mediator of EMT [130, 131]. This suggests that in tumor cells Brachyury might induce EMT through activation of Snail and its downstream genes. Another conserved regulatory interaction occurs between Brachyury and FGF factors, as demonstrated by the Brachyury/FGF autoregulatory loops seen in *Xenopus* and zebrafish [109, 132, 133] and by the binding of human Brachyury to the *FGF8* promoter region [129]. A link between FGF and Brachyury has also been found in ascidians; in *H. roretzi*, the FGF/MEK/MAPK/Ets

signaling pathway is required for the initial expression of *Hr-Bra* through the binding of Ets to the 5′-flanking region of this gene [134]. Similarly, in early *Ciona* embryos, FGF9/16/20 promotes the activation of *Ci-Bra* expression, while subsequently FGF8/17/18, together with FGF9/16/20, ensures its maintenance [135].

The identity of Brachyury target genes in humans has been recently revealed by transcriptome and ChIP-Seq analyses in the chordoma cell line U-CH1 [136]. This study revealed that a large subset of Brachyury targets is involved in cell cycle control, including NUSAP1 and BUB1, spindle checkpoint genes involved in cell proliferation [137, 138]. Another major division of chordoma Brachyury targets is represented by genes encoding extracellular matrix (ECM) components, including *laminin alpha2*, *collagen type VI alpha3*, and *olfactomedin 4* [136]. Lastly, other Brachyury targets of potential medical relevance are chemokines and growth factors, including connective tissue growth factor (CTGF), which has also been reported to control motility and signaling in the notochord [139].

Somewhat surprisingly, a preliminary comparison of the Brachyury targets identified in mouse differentiating stem cells with those found in chordoma does not reveal numerous commonalities. Among the shared Brachyury targets are the Ets-family transcription factor ETV1, which is translocated in certain sarcomas [140], CIT (citron Rho-interacting kinase; [141]), GABRA2 (GABA receptor alpha2), the zinc-finger protein ZDHHC17, which has been previously found to be upregulated in leukemic cells over-expressing Gfi-1B [142], PIGK (phosphatidylinositol glycan, class K), which was found to be downregulated in colorectal cancer due to a polymorphism in its 3′-UTR [143], TNFRSF19 (tumor necrosis factor receptor superfamily, member 19, a.k.a. TROY), previously found to be over-expressed in glial tumors [144], VEPH1, encoding a PH-domain protein expressed in the developing central nervous system [145], PTN (pleiotrophin), which is expressed in the canine notochord and NP [146], SCRG1, encoding an ECM-localized protein involved in chondrogenesis [147], and the cell proliferation activator TGF-alpha, which has been shown to stimulate proliferation of chondrocytes [148]. Interestingly, with the exception of PTN, none of the other genes was previously reported as a possible notochord marker, which likely reflects the fact that cancerous hallmarks are chordoma-specific targets of Brachyury.

7. Possible Mechanisms of Action of Brachyury during Tumorigenesis: Interaction with Other Factors

Brachyury proteins can homodimerize when contacting DNA and bind either palindromic sequences or inverted repeats in this form [82, 106, 107]; in addition, Brachyury can also bind DNA and activate transcription as a monomer [108–110]. The homodimerization of mouse Brachyury is mediated by a short peptide, PESPNF, which was also found to allow its interaction *in vitro* with Brachyury proteins from other species, such as *Drosophila* Brachyenteron [112]. These findings have led to the hypothesis that Brachyury might be able to form heterodimers with other T-box proteins [149].

In terms of non-T-box proteins, Brachyury has been shown to interact with Smad1, a component of the BMP signaling pathway, through a short N-terminal peptide; this region is found only in Brachyury proteins from bilaterian animals [150, 151]. The interaction with Smad1 was shown to restrict the inductive ability of the bilaterian Brachyury proteins to mesoderm formation in *Xenopus* animal cap assays [150]. While the evolutionary significance of this interaction has been clarified, it is still unknown whether this and/or additional protein-protein interactions might also modulate the oncogenic properties of Brachyury.

Another protein that interacts directly with Brachyury is the homeodomain transcription factor Mixl1. *In vitro* studies revealed that these proteins were associated *via* their respective DNA-binding domains [152]. In particular, the T-domain, contained within the first 230 amino acids of the N-terminal region of Brachyury, interacts with helix III of the Mixl1 homeodomain, preferentially when Mixl1 is in its homodimeric form [152]. This interaction with Brachyury does not impede Mixl1 binding to DNA, suggesting that Brachyury might be recruited to DNA by DNA-bound Mixl1 and, thus, regulates some of its target genes without directly binding DNA [152]. Interestingly, the same study proposes that the interaction of the Brachyury N-terminal region with Mixl1 might expose the C-terminal repression domains of Brachyury, leaving them available for interaction with transcriptional corepressors. A similar model has been brought forth in a study of the Ripply proteins, which act as adaptors and recruit the corepressor Groucho/TLE to T-box proteins, shifting their transcriptional activity towards repression [153]. Although a direct interaction with Ripply1 has so far only been shown in the case of Tbx24, another T-box transcription factor related to Brachyury, it is possible that a similar interaction might occur with Brachyury as well. Indeed, Ripply1 is able to suppress the activating ability of Ntl in zebrafish [153].

Even though a physical interaction between Brachyury and members of the Fox subfamily of winged-helix transcription factors has not been reported, it is noteworthy that these transcription factors have been found to work synergistically in different model systems. For example, in *Xenopus*, animal caps from embryos coinjected with *pintallavis*, a member of the Foxa4 class, and *Xbra* mRNAs give rise to mesoderm and notochord [154]. In *Drosophila*, *brachyenteron* (*byn*) and *forkhead* (*fkh*) cooperatively specify the caudal visceral mesoderm, which is absent in *byn*/*fkh* double mutant flies and is formed ectopically only when both genes are over-expressed [101]. In the mouse embryo, both *Brachyury* and *Foxa2* have been reported to be upstream of the homeobox gene *Noto*, based upon the evidence that this gene is down-regulated in mutant embryos for either gene [155]. Finally, studies in ascidians have identified a compact notochord *cis*-regulatory module whose activity depends on both Ci-Bra and Ci-FoxA2 binding sites, thus providing the first report of a synergistic interaction between these factors within a compact regulatory region [156].

Together these studies suggest that the various interactions of Brachyury with other proteins exert a considerable influence on its function, suggesting that their impairment could be both a cause and a consequence of tumorigenesis.

8. Possible Mechanisms of Action of Brachyury during Tumorigenesis: Dosage-Dependent Effects

We have previously discussed the duplication of the *Brachyury* locus as a hallmark of hereditary chordoma. It is important to note, however, that duplications of *Brachyury* have occurred in various species along the phylogenetic tree without negative consequences. For example, two *Brachyury* genes have been identified in the cnidarian *Hydra vulgaris*. Both genes are expressed in the hypostome, although *HyBra1* is expressed predominantly in endoderm, and *HyBra2* in ectoderm [157]. In *Xenopus* animal cap assays, only *HyBra1* is capable of inducing mesoderm formation, similarly to vertebrate Brachyury proteins [157]. In basal chordates, there is only one copy of *Brachyury* in *Ciona* and other tunicates [89, 135], while the cephalochordate amphioxus has two copies, likely the result of a lineage-specific duplication [90]; both amphioxus genes are expressed in the mesoderm and subsequently in the differentiating notochord [158]. In vertebrates, two *Brachyury* orthologs have been found in zebrafish, the well-characterized *no tail*, and its paralog *Brachyury* (recently renamed *ntl-a* and *ntl-b*, respectively); combined inactivation of these genes exacerbates the *ntl-a* mesodermal phenotype, indicating that these factors act synergistically [159]. Also, in mouse, another *Brachyury* gene, *Brachyury the Second* (*T2*), has been found and mapped to the proximal region of chromosome 17 near *Brachyury*; a mutation in this gene reportedly causes defects in notochord formation, although not as severe as those seen in *Brachyury* mutants [160]. *T2* does not seem to be present in the human genome.

Therefore, it appears that duplicated copies of *Brachyury* genes can become functional paralogs, and either segregate into different embryonic territories or continue to be expressed within the same tissue; in the latter case, they may act synergistically and likely control shared target genes. Nevertheless, this partial or complete functional redundancy is expected to require strict tissue-specific regulation in order to prevent the damage that an excessive level of Brachyury protein could cause. In fact, the increased dosage of Brachyury has been shown to exert strong effects on development, as seen in transgenic mouse embryos where three copies of this gene resulted in an extension of the anterior-posterior axis [161]. The main molecular mechanism underlying this effect is likely represented by the dose-dependent binding of Brachyury to the *cis*-regulatory regions of its target genes. An example of this mechanism has been documented in the case of the *Drosophila* gene *orthopedia* that, as mentioned above, is controlled in a dose-dependent fashion by Brachyenteron through multiple binding sites [112]. It has recently been suggested that this mode of gene regulation, whereby Brachyury proteins utilize modular arrangements of canonical and noncanonical binding sites to control some of their targets, might be employed by mouse Brachyury as well [129]. In conclusion, the levels of functional Brachyury protein modulate the function of this transcription factor, most likely by altering the regulation of its direct target genes.

9. Possible Mechanisms of Action of Brachyury during Tumorigenesis: Upstream Regulators of *Brachyury* Transcription

While the identities of Brachyury-downstream genes continue to be elucidated, in parallel, several groups have studied the regulation of the expression of *Brachyury* itself using different model systems. The emerging consensus is that the essential signaling pathways that govern body plan formation and mesoderm induction, Wnt/β-catenin, TGF-β/Nodal/activin, BMP, and FGF, also act upstream of *Brachyury* in concert with different combinations of early-onset transcription factors. The involvement and cross-interactions of each of these pathways vary among different animals. In zebrafish, a Nodal morphogen gradient activates expression of both *Brachyury* orthologs, *ntl-a* and *ntl-b*; however, transcription of *ntl-a* is additionally regulated by both Wnt and BMP signaling, while transcription of *ntl-b* is not responsive to these pathways [162]. The BMP signaling pathway and zygotic Wnt are also upstream of *Xbra* in *Xenopus* [163, 164]. In humans, BMP-4 has been indicated as a regulator of *Brachyury* transcription, being able to induce its expression in hES cells; the same study showed that activin-A and Wnt3a also exhibited minor inducing ability [165].

In some instances, differences in the regulation of *Brachyury* expression are observed between different regions of the notochord in the same animal. For example, in the ascidian *Ciona*, the first step leading to the activation of *Brachyury* expression in the main notochord lineage (the 32 anterior notochord cells, or A-lineage) is the translocation of cytoplasmic β-catenin to the nuclei in vegetal blastomeres, an event that activates transcription of *FGF9/16/20* and of the transcription factor FoxD [166]. In turn, FoxD activates expression of the zinc-finger transcription factor ZicL that, along with FoxD, binds the *Brachyury* notochord CRM and activates transcription [167]. However, in the posterior-most 8 notochord cells (secondary notochord, or B-lineage), *Brachyury* expression is instead regulated by Nodal and Delta-like [168].

Although the signaling pathways upstream of *Brachyury* overall appear to be conserved across the phylogenetic tree, the transcription factors that control expression of this gene vary among different organisms. In *Ciona*, Ci-FoxA2 has also been reported to activate *Brachyury* expression [169], and this might be the case in other organisms where the expression of *Foxa2* precedes that of *Brachyury* genes. The finding that the mouse *Foxa2* promoter region is bound by Brachyury suggests that the two genes might be regulating each other [129]. In addition to mesoderm inducers, a factor mainly known for its involvement in innate and adaptive immune response, NF-kappaB, has been shown to modulate

the transcription of *ntl-a* in zebrafish and of *Xbra* in *Xenopus* [170]. Furthermore, it seems reasonable to hypothesize that after their transcription is initiated, by one or more of the aforementioned pathways, Brachyury proteins can positively regulate their own transcription, either directly or indirectly, as is the case for other transcription factors. In fact, autoregulation has been reported in the case of Xbra [171], and ChIP-Seq experiments have shown that human Brachyury binds its own locus [136], although the existence of this autoregulatory loop has been ruled out in mice [172].

Transcription of *Brachyury* is also modulated by repressive interactions. In *Ciona*, expression of *Ci-Bra* is excluded from muscle cells, and thus confined to the notochord, by the zinc-finger transcriptional repressor Snail [173]; in *Xenopus*, *Xbra* is actively silenced by another zinc-finger repressor, Smad-interacting protein (SIP1) [174]. Also in *Xenopus*, Xbra and the homeodomain protein goosecoid repress each other, while transcription of both genes is stimulated by activin [175]. This interaction is evolutionarily conserved, as shown by the finding that *Brachyury* is repressed in goosecoid-overexpressing mouse ES cells [176]. Repression can also take place indirectly, as it is seen in the case of embryonic stem cells, where the Smek/PP4c/HDAC1 complex binds the Tcf/Lef binding site of the *Brachyury* promoter and causes histone deacetylation, thus impeding transcription [177].

10. Concluding Remarks

From the studies that we have summarized above, it seems that Brachyury-expressing cells have the potential to somehow give rise to chordomas, possibly through one or more of the mechanisms that we have outlined. If this is the case, it remains to be determined whether and how normal Brachyury-expressing notochord cells turn into the Brachyury-expressing cells found in chordomas. Possible clues to the solution of this problem might come from a recent gene expression study showing that chordomas contain cancer stem-like cells [178]. In this study, some of the chordoma cells from the U-CH1 cell line were found to express stem cell surface markers. These results provide the first evidence that chordomas might contain cancer stem cells (CSCs) and could open new avenues for research on possible treatments. Intriguingly, recent studies carried out in adenoid cystic carcinoma cell lines indicate that CSC characteristics can be reversed by the knockdown of *Brachyury*, along with the ability of these cells to undergo EMT [179]. Taken together, these recent findings again point at *Brachyury* as a possible therapeutic target. Within this context, the discovery of the immunogenic properties of Brachyury and the subsequent development of specific $CD8^+$ cytotoxic T-cells able to destroy Brachyury-expressing tumor cells from various cells lines *in vitro* [130, 180] represent an encouraging step towards the development of Brachyury-targeted therapies. More generally, therapeutic agents targeting Brachyury could also be employed for cancer immunization strategies directed against any cancer cell undergoing EMT that expresses this marker [130]. Brachyury-targeted therapeutics could possibly be implemented through adaptations of recently developed exosome-based nanodevices, which increase the immunogenicity of tumor-associated antigens [181]. Lastly, new treatments could be directed against one or more of the numerous genes controlled by Brachyury, to increase their specificity.

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Review Article

Hereditary Breast Cancer: The Era of New Susceptibility Genes

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Breast cancer is the most common malignancy among females. 5%–10% of breast cancer cases are hereditary and are caused by pathogenic mutations in the considered reference *BRCA1* and *BRCA2* genes. As sequencing technologies evolve, more susceptible genes have been discovered and *BRCA1* and *BRCA2* predisposition seems to be only a part of the story. These new findings include rare germline mutations in other high penetrant genes, the most important of which include *TP53* mutations in Li-Fraumeni syndrome, *STK11* mutations in Peutz-Jeghers syndrome, and *PTEN* mutations in Cowden syndrome. Furthermore, more frequent, but less penetrant, mutations have been identified in families with breast cancer clustering, in moderate or low penetrant genes, such as *CHEK2*, *ATM*, *PALB2,* and *BRIP1*. This paper will summarize all current data on new findings in breast cancer susceptibility genes.

1. Introduction

Breast cancer is a disease in which breast cells become abnormal and multiply to form a malignant tumor. Breast cancer is the most common form of cancer and the second most common cause of death from a neoplastic disease affecting women. One in 8 women will develop breast cancer in her lifetime in the developed world [1, 2]. There are a number of recognized risk factors for breast cancer development including hormonal, reproductive, and menstrual history, age, lack of exercise, alcohol, radiation, benign breast disease, and obesity [3]. Nevertheless, the key factor to breast cancer development is the early onset of disease. Individual risk increases proportionally with affected relatives with breast cancer and early age of onset [2]. Although approximately 10%–30% of breast cancer cases are attributed to hereditary factors, only 5%–10% of breast cancer cases are identified with a strong inherited component, while only a small fraction of these cases (4%-5%) is explained by mutations in high penetrant genes transmitted in an autosomal dominant manner [4–7].

BRCA1 and *BRCA2* genes are the most commonly mutated genes, but additional genes associated with hereditary breast cancer are emerging [8]. New advances in genomic technologies have led to parallel testing of multiple genes. Customized next generation sequencing panels are now providing the simultaneous analysis of breast cancer predisposition genes, from high- to intermediate-penetrant genes. Nonetheless, some of these genes have also been associated with increased risk of other cancers, such as ovarian, pancreatic, and colorectal cancer.

2. Patient Eligibility

The implementation of hereditary multigene panel testing arises many issues, such as which are the criteria that patients have to meet in order to undergo the test and the patient clinical management. The utilization of the test must be in compliance with the recommendations for genetic testing identified in the ASCO policy [9].

BRCA1 and *BRCA2* negative patients with a personal or family history of hereditary cancer can be eligible for customized gene panel testing. Criteria have been amended from the proposed National Comprehensive Cancer Network (NCCN) guidelines and are summarized in Table 1.

Table 1: Criteria of target population for genetic test on customized gene panel modified from (http://www.nccn.org/).

Individual with breast/ovarian cancer personal history and one of the following:
(i) breast and/or ovarian or pancreatic cancer in at least two blood relatives;
(ii) multiple primary breast cancers or bilateral breast cancer, first diagnosed before the age of 50 years;
(iii) premenopausal triple negative breast cancer diagnosed at a young age (<45 years);
(iv) male breast cancer in a blood relative;
(v) ethnicities with high *BRCA* mutation frequency, such as Ashkenazi Jews, should be tested, even in the absence of family history.

3. Penetrance

Cancer predisposing genes can be categorized according to their relative risk of a particular type of cancer. High-penetrant genes are associated with a cancer relative risk higher than 5. Low-penetrant genes are presented with relative risk around 1.5, whereas intermediate-penetrant genes confer relative cancer risks from 1.5 to 5. All genes described, along with their chromosomal position and the phenotypic features, are summarized in Table 2.

3.1. High-Penetrant Genes

3.1.1. BRCA1. *BRCA1* encodes a nuclear phosphoprotein, which acts as a tumour suppressor gene through maintaining genomic stability [4]. The encoded protein combines with other tumour suppressors, DNA damage sensors, and signal transducers to form a large multisubunit protein complex, known as the *BRCA1*-associated genome surveillance complex [10].

BRCA1 inherited mutations predispose to high risk of breast and ovarian cancers. Lifetime risks of breast and ovarian cancer, are as high as 80% and 40%, respectively, among women carrying *BRCA1* mutations, while they are characterized by elevated cancer risk at younger ages [11, 12]. While mutations are found throughout the gene's coding region, extensive population analyses have led to the identification of founder mutations [13–16]. *BRCA1*-related cancers have distinct pathological features and are generally characterized by the lack of expression of human epidermal growth factor 2, estrogen, and progesterone receptors (triple negative breast cancer) [17].

The recent therapeutic approaches towards *BRCA1* carcinomas have increased the clinical utility of *BRCA1* genetic analysis. Inhibitors of the poly-ADP ribose polymerase (PARP) inhibitors can provide an alternative route in treatment since they can effectively kill *BRCA1*-deficient cells [18, 19].

3.1.2. BRCA2. *BRCA2* gene is involved in the maintenance of genomic stability and more specifically, the homologous recombination (HR) pathway which repairs double-strand DNA breaks.

Male *BRCA2* mutation carriers confer a lifetime risk of prostate, breast, and pancreatic cancers around 20%, 6%, and 3%, respectively. Female *BRCA2* mutation carriers face a lifetime risk around 26%–84% for breast cancer and 20% for ovarian cancer [20–22].

BRCA2 is a large gene comprising of 27 exons and mutations can occur throughout the gene. The majority of mutations are frameshifts, but there are a number of missense mutations of which the pathogenicity is usually unclear (variants of unclassified significance-VUS). *BRCA2*-related tumours usually express estrogen and progesterone receptors and tend to have similar features to sporadic breast cancers, unlike *BRCA1*-related cancers [23–25].

According to the 2007 ACS guidelines, individuals carrying pathogenic *BRCA* mutations should undergo a particular surveillance protocol. Annual breast cancer imaging by mammography and/or magnetic resonance imaging (MRI), which is generally a more sensitive detection method, is recommended from the age of 30 [26]. Prophylactic surgeries that include bilateral mastectomy and salpingo-oophorectomy can significantly reduce mortality in these patients [27, 28]. Chemoprophylaxis, such as tamoxifen administration, can also be an alternative route in hormone-dependent tumours [29].

A major limitation of *BRCA1* and *BRCA2* genetic testing is the number of inconclusive results due to variants of unknown significance (VUSs). VUSs are mainly missense and splice site mutations or can be even silent variants.

The interpretation of such variations can be difficult for physicians and problematic for individuals. The approach towards the evaluation of a VUS variant can be multifactorial, involving the *in silico* analysis, where specified software is used to predict the phylogenetic conservation and the protein modification caused. Additionally, segregation analysis of the variant with the disease is the main clarification for the pathogenicity of the variant. VUSs with clear data towards pathogenicity require special attention and specialized prevention strategies.

Splicing is an important mechanism during which accurate removal of introns is taking place in pre-mRNA molecules. Apart from the classical splice site sequences, exonic splice enhancers (ESEs) seem to be crucial for correct splicing. ESEs are short (6–8 nucleotides long) exonic motifs that serve as binding sites for specific serine/arginine-rich proteins [30].

Disruption of ESEs sequences, which can occur in the case of missense mutations or even silent polymorphisms, can result in exon skipping and, therefore, in the production of an alternate, possibly not being fully functional, gene product. Four ESEs, responsive to serine/arginine-rich proteins (SF2/ASF, SC35, SRp40, and SRp55), have been identified in the mammalian cell [31]. ESE motifs, which are scattered throughout the genome, play an important role in exon recognition. A human exon can contain several such motifs, some of which may not be functional [32]. The disruption of these ESEs, which can be caused by synonymous or nonsynonymous genetic variants, can cause

Table 2: Breast cancer susceptibility genes.

Syndrome	Gene or locus (chromosomal location)	Neoplasm	Lifetime risk
Genes with high-penetrance mutations			
Hereditary breast/ovarian cancer syndrome	*BRCA1* (17q12–21)	Female breast, ovarian cancer	40–80%
	BRCA2 (13q12-13)	Male and female breast, ovarian, prostate, and pancreatic cancer	20–85%
Li-Fraumeni syndrome	*TP53* (17p13.1)	Breast cancer, sarcomas, leukemia, brain tumours, adrenocortical carcinoma, lung cancers	56–90%
Cowden syndrome	*PTEN* (10q23.3)	Breast, thyroid, endometrial cancer Other: benign hamartomas, macrocephaly	25–50%
Peutz-Jeghers syndrome	*STK11* (19p13.3)	Breast, ovarian, cervical, uterine, testicular, small bowel, and colon carcinoma Other: Hamartomatous polyps of the small intestine, mucocutaneous pigmentation	32–54%
Hereditary gastric cancer	*CDH1* (16q22.1)	Hereditary diffuse gastric, lobular breast, colorectal cancer	60%
Moderate-penetrance mutations			
ATM- related	*ATM* (11q22.3)	Breast and ovarian cancers	15–20%
CHEK2- related	*CHEK2* (22q12.1)	Breast, colorectal, ovarian, bladder cancers	25–37%
PALB2-related	*PALB2* (16p12.1)	Breast, pancreatic, ovarian cancer, male breast cancers	20–40%
Moderate risk breast/ovarian cancer	*BARD1* (2q34-q35), *BRIP1* (17q22–q24), *MRE11A* (11q21), *NBN* (8q21), *RAD50* (5q31), *RAD51C* (17q25.1), *XRCC2* (7q36.1), *RAD51D* (17q11), *ABRAXAS* (4q21.23)	Breast and ovarian cancers	variable

the failure of the serine/arginine-rich proteins to bind to the ESE motifs and cause exon skipping. ESEs can be initially assessed by available *in silico* tools [33], but can only be confirmed experimentally by RT-PCR. Furthermore, *in silico* data should be treated with caution, since a number of studies have failed to confirm experimentally the initial findings [34, 35].

A major limitation of *BRCA1* and *BRCA2* genetic testing is the number of inconclusive results due to unclassified sequence variants. A fraction of variants of unclassified significance (VUS) can be determined deleterious, if they lie within ESE motifs and can, therefore, explain the genetic factor in families with family history [35–37].

In many cases, the mutated ESEs might not lead to fully functional transcripts, or even the transcripts produced might be underrepresented, so their actual contribution to pathogenicity can be unclear [38].

3.1.3. TP53. *TP53* is a tumour suppressor gene that causes Li-Fraumeni syndrome and affects adults and children. This highly penetrant gene predisposes for a wide spectrum of tumours, including sarcomas, adrenocortical carcinomas, brain cancer, and very early onset breast cancer [39, 40]. Most cancers are manifested from birth through late adulthood [39]. *TP53* mutation carriers face a lifetime cancer risk that exceeds 90% [40–42], while the clinical benefit of extensive surveillance of these individuals remains uncertain [43].

Patients with Li-Fraumeni syndrome have an abnormal response to low-dose radiation that should be avoided as a therapeutic approach because of the increased secondary tumour risk [44].

Breast cancer is the most frequent malignancy among female *TP53* mutation carriers, with approximately 5% of these cases being diagnosed before the age of 30 [39]. While Li-Fraumeni syndrome accounts for a small fraction of breast cancer cases (~0.1%), *TP53* mutation carriers have from an 18- to 60-fold increased risk for early onset breast cancer (diagnosed before the age of 45) when compared to the general population [45–48].

3.1.4. PTEN. Germline mutations in the tumour suppressor *PTEN* gene are the cause of Cowden syndrome. Cowden syndrome is an autosomal dominant disorder characterized by multiple hamartomas with a high risk of benign and malignant tumours of the thyroid, breast, and endometrium. Mucocutaneous lesions, thyroid abnormalities, fibrocystic disease, multiple uterine leiomyoma, and macrocephaly can also be seen. Affected individuals have a lifetime risk up to 50% for breast cancer, 10% for thyroid cancer, and 5–10% for endometrial cancer. Over 90% of individuals with Cowden

syndrome will express some clinical manifestation by their 20s [49–53].

3.1.5. STK11. Germline mutations in the serine/threonine kinase gene (*STK11*/*LKB1*), a tumour-suppressor gene important for mediation of apoptosis and cell cycle regulation, cause Peutz-Jeghers syndrome. Peutz-Jeghers syndrome is an autosomal dominantly inherited syndrome characterized by mucocutaneous pigmentation and hamartomatous polyposis [54]. In addition to an elevated risk of gastrointestinal cancers, an increased risk of cancers at other sites, such as breast [55], small bowel, pancreas, ovary, uterus, stomach, cervix, lung, and testis, has been described [56–61].

STK11 mutation carriers confer a high cumulative risk of any cancer (up to 85%) [62]. In terms of surveillance, Peutz-Jeghers patients should undergo gastrointestinal endoscopy starting from early teens and annual breast MRI starting, at the age of 25–30 [56].

3.1.6. CDH1. The E-cadherin gene (*CDH1*) is a calcium-dependent cell-cell adhesion molecule expressed in junctions between epithelial cells [63]. *CDH1* germline mutations have been associated with hereditary diffuse gastric carcinoma, often with signet ring cell histology. Patients with germline *CDH1* mutations carry an increased risk of lobular breast cancer and colorectal cancer [64, 65]. The cumulative risk of gastric cancer in male and female mutation carriers is approximately 67% and 83%, respectively, with a mean age of diagnosis of 40 years [64]. Moreover, women carriers face a 40%–54% lifetime risk of developing lobular breast cancer [66, 67].

Mutations in *CDH1* are the genetic cause of up to 48% of the diffusion gastric cancer kindreds [68], while in contrast to other cancer predisposition syndromes, splice-site and missense mutations are common, suggesting that even reduced E-cadherin expression can be deleterious [69].

3.2. Moderate Penetrant Genes

3.2.1. CHEK2. Checkpoint kinase 2 (*CHEK2, Chk2*), the protein product of the *CHEK2* gene, is a serine threonine kinase that is activated in response to DNA damage and plays an important role in transducing the DNA damage signal to downstream repair proteins [70]. CHEK2 protein structure shows three characteristic domains: an N-terminal SQ/TQ cluster domain, a forkhead-associated (FHA) domain, and a serine/threonine kinase domain.

Certain mutations in *CHEK2* are reproducibly associated with increased risks of female breast cancer [71]. A particular germline mutation, *CHEK2* c.1100delC, has been shown to increase breast cancer risk 2-fold [72]. While it seems to be quite frequent (~3%) in northern European populations (Finish, Dutch) [42, 73], it is rather rare (~0.5%) in southern European populations [74]. Carriers of the *CHEK2* c.1100delC mutation have an increased risk of bilateral breast cancer and male breast cancer [75]. A recent study described families with homozygous *CHEK2* c.1100delC mutations. Women homozygous for the mutation have a sixfold higher risk of breast cancer when compared to heterozygotes [76].

Another *CHEK2* variant, *CHEK2* p.I157T, which is located in exon 3 of the gene, is associated with lower breast cancer risk (~1.5) [74, 77]. There is also an increased risk of other malignancies within families carrying *CHEK2* mutations including colon, prostate, kidney, and thyroid cancer [78].

Remarkably, many identified rare variants include missense genetic alterations whose functional consequences are rather difficult to assess. *In vivo* DNA damage assays [79] that can determine their activity can accompany segregation and *in silico* analyses to determine the pathogenicity of these variants.

3.2.2. PALB2. *PALB2*, also known as *FANCN*, is a Fanconi anemia gene that encodes for a protein that interacts with *BRCA2* during homologous recombination and double-strand break repair. It confers breast and ovarian cancers susceptibility [80]. Casadei et al. sequenced *PALB2* in high-risk breast cancer families, identifying *PALB2* mutations in 33 of 972 families (3.4%) [81]. It is worthwhile to mention that 18 of these 33 families (55%) had a family member with ovarian cancer, who was confirmed to carry the familial *PALB2* mutation. Notably, these families had a similar phenotype to *BRCA2*, with an increased incidence of pancreatic as well as breast and ovarian cancers. Familial pancreatic and/or breast cancer due to *PALB2* mutations is inherited in an autosomal dominant pattern, while Fanconi anaemia is an autosomal recessive condition [82, 83].

In another study, rare germline mutations in *PALB2* were identified among patients with breast cancer. The first-degree female relatives of these carriers demonstrated significantly higher incidence of breast cancer than relatives of noncarriers, indicating that pathogenic *PALB2* mutations confer an estimated 5.3-fold increase in risk. Moreover, the overrepresentation of mutations in the cohort of women with contralateral breast cancer is important to the clinical management of women carrying *PALB2* mutation as it implies a significant risk of developing a second primary breast neoplasm [84]. Dansonka-Mieszkowska et al. identified a Polish *PALB2* founder mutation in 0.6% of individuals with ovarian carcinoma but only in 0.08% of healthy controls. This mutation was further studied on groups of sporadic and familial breast cancer patients and healthy controls and was estimated that it can increase the risk of familial breast cancer [85].

Recently, *PALB2* was reported to be a new pancreatic cancer susceptibility gene as truncating mutations were identified in American patients with familial pancreatic cancer. Mutations in *PALB2* were also detected in European families and, interestingly, each of these had also a history of breast cancer [83]. *PALB2* mutation carriers of familial pancreatic cancer have to be considered as high-risk individuals with at least 10- to 32-fold increased risk depending on the number of affected family members [86]. Such high-risk family members should be offered screening programs for the early detection and potentially curative operative treatment

of pancreatic cancer [86], as it has been shown that it can be effective [87, 88].

3.2.3. ATM. The protein deliverable of the *ATM* gene is a PI3 K-related protein kinase [89]. ATM has multiple complex functions, including a central role in the repair of DNA double-strand breaks, a pathway that includes TP53, BRCA1, and CHEK2 proteins [90].

It is proposed that *ATM* mutation heterozygotes have a 2-fold higher breast cancer risk compared to the general population. This risk is elevated 5-fold in women under the age of 50 [91]. The gene's penetrance is approximately 15%, while accurate prediction of who of these mutation carriers will develop breast cancer is not feasible.

It is difficult to assess the clinical utility of genetic testing for *ATM* at present. However, these *ATM* mutation carriers may merit different approaches to treatment for breast cancer due to their increased radiosensitivity or efficacy of specific chemotherapies associated with *ATM* mutations [92].

Homozygous or compound heterozygous *ATM* mutations cause ataxia telangiectasia, which is characterized by progressive cerebellar ataxia, oculomotor apraxia, immunodeficiency, and general increased risk of malignancies [93]. Lymphoid cancers predominate in childhood, and epithelial cancers, including breast cancer, are seen in adults [94].

3.2.4. BRIP1. *BRIP1* encodes a protein that was identified as a binding partner of *BRCA1* and was investigated as a breast cancer predisposing gene. In 2006, truncating mutations were identified in breast cancer families [95], while the relative breast cancer risk, although there are reports of higher risks in some families, was estimated around 2. *BRIP1* germline mutations also confer an increased risk of ovarian cancer [96].

Recently, three *BRIP1* missense mutations have been identified in high-risk Jewish women, who have been tested negative for mutations in *BRCA1* and *BRCA2* genes, indicating that *BRIP1* mutations can contribute to breast cancer susceptibility in Jewish high-risk families [97]. Moreover, rare *BRIP1* mutations have been identified in Spanish and Icelandic ovarian kindreds, indicating that *BRIP1* behaves like a classical tumor suppressor gene in ovarian cancer [96]. Biallelic mutations of *BRIP1* cause Fanconi anemia complementation group J, a phenotype different to that caused by biallelic mutations in *BRCA2,* resulting in much lower rate of childhood solid tumours [2].

3.2.5. RAD51C. *RAD51C* is an essential gene in homologous recombination, while biallelic missense mutations in the gene cause a Fanconi anemia-like phenotype [98]. *RAD51C* was investigated as a possible breast and ovarian cancer susceptibility gene in 1100 high-risk families, who were previously tested negative for *BRCA1* and *BRCA2* mutations. Germline mutations were identified in 1.3% of families with both breast and ovarian cancers, with a mean age of diagnosis of 53 and 60 years, respectively. No pathogenic mutations were identified in families with breast cancer cases only [99]. In a subsequent, but larger, Finnish study, *RAD51C* mutations were identified in ovarian cancer families only [100], while in a recent Spanish study, identified *RAD51C* mutations in 1.3% of breast and ovarian cancer families, with mutations in families with breast cancer cases only, were rare [101]. The inclusion of *RAD51C* gene in routine clinical testing is a controversial matter, mainly due to its low incidence or lack of mutation identification in particular populations.

3.2.6. XRCC2. *XRCC2* is a *RAD51* paralog and plays an important role in the homologous recombination pathway that repairs double-strand breaks. Failure of these processes will lead to mutations, and as a result *XRCC2* might be responsible for cancer predisposition and especially a breast cancer susceptibility gene [102, 103].

An initial exome-sequencing study identified two germline *XRCC2* mutations, while a larger-scale genetic analysis revealed ten rare *XRCC2* variants in breast cancer families, some of which were definitely pathogenic [104].

Another study suggested that some *XRCC2* coding SNPs can influence breast cancer risk and survival. Particularly, the specific *XRCC2,* p.R188H missense mutation was associated with poor survival prognosis [104].

On the contrary, Hilbers et al. failed to identify unique variants in familial breast cancer patients only, questioning the cancer susceptibility of *XRCC2* gene. The only predicted deleterious variant was detected in a control individual, while missense variants were evenly distributed in patients and controls. Although a small relative risk can be attributed to *XRCC2* mutations, the actual association needs further evaluation [102, 105].

Since *XRCC2* gene is a key mediator in homologous recombination pathway, *XRCC2* mutation carriers may benefit from specific targeted therapies such as PARP-inhibitors, but the actual influence of *XRCC2* mutations on breast cancer susceptibility requires further investigation.

3.2.7. NBS1, RAD50, and MRE11. The MRE11-RAD50-NBS1 (MRN) protein complex plays an important role in sensing and early processing of double-strand breaks, thus maintaining genomic integrity [106, 107]. This protein complex integrates DNA repair with checkpoint signalling through the ATM, BRCA1, and CHEK2 proteins [106]. Based on the complex's important role in preventing malignancies, a number of studies have screened breast and/or ovarian cancer families for germline mutations in the coding regions of the aforementioned genes. Potentially pathogenic mutations have been identified in all three genes. Specifically, *MRE11* and *NBS1* mutations in highly conserved amino acids that have not been identified in controls have been described in Finish high-risk families [107, 108]. In respect to *RAD50,* a relatively common low-risk allele was identified in patients and controls, as well as a small number of unique rare pathogenic alleles. The interesting finding is the increased genomic instability in peripheral blood T-lymphocytes drawn from these mutation carriers [106]. Analyzing breast cancer patients' tumours can lead to the identification of *MRE11* germline mutations based on the reduced or lack of expression of all three (MRN) proteins [109]. *NBN* mutation carriers confer elevated risks

for a numerous types of cancers, including breast cancer [8, 106, 108, 110–112], which can be estimated to a 2- to 3-fold increase [110], while family relatives display a higher rate of various forms of cancers [112, 113].

Even minor disturbances of complexes' activity have profound effects on the genomic integrity and, thus, all three components have been implicated in recessive genetic instability disorders. More importantly, individuals carrying biallelic hypomorphic *NBN* mutations suffer from the Nijmegen breakage syndrome, being susceptible to several types of cancer. Approximately 40% of them will develop a malignancy before the age of 21 [110].

Germline mutations in *NBS1, RAD50,* and *MRE11* genes, although seen in low frequencies and can be population specific, can be qualified as novel candidates for breast cancer susceptibility in a subset of non-*BRCA1* and *BRCA/2* families. However, their clinical impact is yet to be determined.

3.2.8. BARD1. BARD1 (*BRCA1*-associated RING domain) was identified initially as a protein interacting with BRCA1 in DNA double-strand break repair and apoptosis initiation. *BARD1* mutations have been detected in breast, ovarian, and endometrial cancers. Initial *BARD1* mutational analysis in familial and sporadic cases revealed four different germline mutations not followed by subsequent loss of heterozygosity [114]. More recent studies have been successfully identified *BARD1* mutations in high-risk families [8, 115]. *BARD1* mutations can confer cancer susceptibility, but larger studies are essential to confirm that.

3.2.9. ABRAXAS. ABRAXAS (also known as *ABRA1*, *CCDC98,* or *FAM175A*) codes a protein that is an essential component of BRCA1 holoenzyme complex as it binds to *BRCA1* BRCT motifs via its phosphorylated C-terminus. *Abraxas* as well as the other members of this complex (*RAP80*, *BRCC36*, *BRCC45,* and *MERIT40/NBA1*) is involved in DNA damage checkpoints in response to double-strand breaks.

Recently, proteomic analysis revealed the binding of ABRAXAS to the BRCA1 BRCT (BRCA1 carboxyl-terminal) repeats, which are essential elements in tumour suppression. Due to the close interaction to *BRCA1*, *Abraxas* might be a cancer susceptibility gene and might play a role in hereditary breast and ovarian carcinoma [116].

Although there is only a small number of studies, *Abraxas* constitutes a good candidate for yet unexplained cases with strong family history. A missense alteration, p.R361Q, resulting in abnormal DNA response, was identified in 3 out of the 125 Finish, *BRCA1* and BRCA2 negative, families and one out of the 991 unselected breast cancer cases studied. The missense allele segregated with the disease in the two families, while no *Abraxas* genetic alterations were identified in the healthy controls studied [117].

Therefore, based on these preliminary data, *Abraxas* can be considered as a new breast cancer susceptibility gene.

3.2.10. RAD51D. RAD51D is one of the five paralogs of RAD51 protein family. RAD51 family members are similar to bacterial RecA and *Saccharomyces cerevisiae* Rad51, which are known to be involved in DNA repair pathway. Its gene product complexes with other RAD51 protein members, while it is an important element in homologous recombination in the eukaryotic cells along with other gene products [118, 119].

Loss-of-function mutations in *RAD51D* gene seem to predispose to ovarian cancer, while there is doubtable association to breast cancer susceptibility. *RAD51D* pathogenic mutations are generally rare, contributing to approximately 0.5%–0.9% of breast/ovarian probands of *BRCA1* and *BRCA2* negative families [120, 121]. Another study successfully identified deleterious *RAD51D* mutations in 0.8% of unselected patients previously diagnosed with ovarian, peritoneal, or fallopian tube cancer [122]. Interestingly, there seems to be a higher prevalence of *RAD51D* mutations in families where there is elevated ovarian cancer burden (2 or more ovarian cancer cases) [120, 121].

The ovarian cancer relative risk for carriers of *RAD51D* mutations is estimated to be 6.3, while the relative risk for breast cancer is not statistically significant [120]. A single *RAD51D* splice mutation has been identified to have founder effect within the Finnish population [123].

PARP inhibitors can be considered as a therapeutic alternative for *RAD51D* mutation carriers, as RAD51D-deficient are sensitive to PARP inhibitors [120].

4. Low Penetrant Breast Cancer Loci

A number of common breast cancer susceptibility loci have been associated with a slightly increased or decreased risk of breast cancer. These can follow the polygenic model, or can act synergistically with environmental factors or lifestyle, to account for a small fraction of familial breast cancer cases.

Most of these low-susceptibility loci have been highlighted through genome wide association studies (GWAS) and initially included a number of loci. In the final breast cancer assessment risk, six SNPs showed statistically significant association: *MAP3K1*, *FGFR2*, *LSP1*, *TNRC19,* and *H19* [124–128].

Moreover, a particular SNP in *CASP8* was identified to confer a slightly increased susceptibility in a candidate-gene study [129, 130].

Although the actual contribution of low power, common susceptibility loci in hereditary breast cancer is debatable, the identification of such alleles can explain a subset of breast cancer cases.

5. Benefits of Genetic Testing

The knowledge of a patient's genetic susceptibility for breast cancer can orientate appropriately clinical management. This information can provide the following options.

(i) Modify breast cancer surveillance options and age of initial screening.

(ii) Suggest specific risk-reduction measures (e.g., consider prophylactic salpingo-oophorectomy after

childbearing and/or prophylactic bilateral mastectomy, for women with increased risk for breast and/or ovarian cancer).

(iii) Clarify familial cancer risks, based on gene-specific cancer associations.

(iv) Offer treatment guidance (e.g., avoidance of radiation-based treatment methods for individuals with a *TP53* mutation).

(v) Identification of other at-risk family members.

(vi) Provide customized, gene-specific, treatment options (e.g., PARP-inhibitors in *BRCA1*-mutation carriers).

(vii) Preimplantation diagnosis.

6. Future Perspectives

In the last few years a significant progress has been made in broadening the spectrum of cancer-related genes. The potentials of new sequencing technologies, from whole genome to exome sequencing, can accelerate the discovery of new susceptibility genes, not only for breast cancer, but also for other cancers too. Targeted capture and massively parallel sequencing of specific genes can successfully identify families at risk for developing breast and/or ovarian cancer, while it seems that this technique is now ready to be applied in a clinical setting. Knowing the genetic defect can provide the route to customized, targeted therapies with extremely beneficial results. Nevertheless, this era of new genes while opening new roads in cancer susceptibility still needs to be treated with caution. Genetic counseling for most of these new genes can be complicated, while extreme prevention strategies, such as prophylactic surgeries cannot be recommended with current data. Further evaluation and genetic analysis in large series of patients will determine actual cancer risks.

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Review Article

The Role of MicroRNAs in Cancer Susceptibility

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Single nucleotide polymorphisms (SNPs) are germline variations interspersed in the human genome. These subtle changes of DNA sequence can influence the susceptibility to various pathologies including cancer. The functional meaning of SNPs is not always clear, being, the majority of them, localized in noncoding regions. The discovery of microRNAs, tiny noncoding RNAs able to bind the 3′ untranslated region (UTR) of target genes and to consequently downregulate their expression, has provided a functional explanation of how some SNPs positioned in noncoding regions contribute to cancer susceptibility. In this paper we summarize the current knowledge of the effect on cancer susceptibility of SNPs included in regions related with miRNA-dependent pathways. Hereditary cancer comes up from mutations that occur in high-penetrant predisposing tumor genes. However, a considerable part of inherited cancers arises from multiple low-penetrant predisposing gene variants that influence the behavior of cancer insurgence. Despite the established significance of such polymorphic variants in cancer predisposition, sometimes their functional role remains unknown. The discovery of a new group of genes called microRNAs (miRNAs) opened an avenue for the functional interpretation of polymorphisms involved in cancer predisposition.

1. Biogenesis of MicroRNAs

MicroRNAs are an ample class (more than one thousand) of small (19–25 nucleotides) noncoding RNAs that downregulate the expression of target genes, binding mainly their 3′ untranslated region (3′UTR). Genomic regions containing microRNA genes are transcribed by RNA Polymerase II, and large RNA precursors, named pri-miRs (primary miRNAs), are generated [1]. The Pri-miRs are processed by a multiprotein complex in which the two core elements are Drosha, an enzyme with ribonuclease III activity, and Pasha (also known as DGCR8) an RNA-binding protein [2]. This processing results in the formation of a second precursor, called pre-miR, of ~70 nucleotides having a stem-loop structure with imperfect base pairings. The pre-miR is then exported by the complex Exportin5/RanGTP into the cytoplasm, where Dicer, a protein with ribonuclease III activity, cuts the RNA stem-loop structure leading to the formation of an miRNA duplex [1]. One strand of the duplex is selected, for thermodnamical reasons, to yield the mature miRNA while the other strand is degraded. Finally, the mature microRNA is loaded in a ribonuclease complex called RISC (RNA-induced silencing complex), containing the Argonaut (Ago 1–4) proteins and the GEMIN3 and GEMIN4 factors, where it pairs with the 3′UTR of target genes. The matching of miRNAs with their target genes causes the mRNA degradation or the inhibition of mRNA translation [3].

Single nucleotide polymorphisms (SNPs) related to miRNA genes, miRNA binding sites, or in genes of the miRNA processing machinery can affect the final level and function of miRNAs. This distinctive and relative new group of polymorphisms is called miRSNPs.

Target genes of microRNAs can be also oncogenes [4, 5] or tumor suppressor genes [6–8], and, hence, microRNAs are

important players in carcinogenesis [9]. The presence of SNPs either in the genomic miRNA sequences or in the 3′UTR of cancer-related genes could influence miRNA-dependent regulation altering consequently tumor susceptibility (Figure 1) [10]. Here, we review miRSNPs studied for their relevance in the susceptibility to human cancer, indicating the associated risk expressed as odds ratio (OR) with 95% confidence interval (95% CI) (Table 1).

2. SNPs in MicroRNA Genomic Regions

SNPs can span in the entire region of the pri-miR affecting the different steps of microRNA processing or the pairing with the 3′UTR of target genes. Therefore, the expression or the sequence of the mature miRNA could be impaired, and the regulation of all gene targeted by a given miRNA could be consequently affected.

Several SNPs in microRNA regions are currently intensively investigated in different cancer types but in some cases their significance remains to be established and further studies are required to clarify their possible role in cancer predisposition.

The SNP rs2910164 is located in the pri-miRNA sequence of miR-146a and has C and G allelic forms. It was first studied by Jazdzewski et al. whom found a significant different distribution of genotypes, in patients with papillary thyroid carcinomas compared with normal subjects, being the GC genotype associated with an increased risk of papillary thyroid carcinoma (OR = 1.62; 95% CI: 1.3–2.0; $P = 0.000007$) [11]. Furthermore, the authors demonstrated that in heterozygotes the expression of mature miR-146a is reduced. Wang et al. showed that the C allele of SNP rs2910164 is associated with significantly decreased risk of bladder cancer (OR = 0.80; 95% CI: 0.71–0.90), and GC/CC genotypes confer a significantly reduced risk of recurrence, compared with the GG genotype. The same authors demonstrated by functional studies that the miR-146a rs2910164 C allele inhibits proliferation in bladder cancer cells [12]. In a case-control study, Lung et al. showed that CC genotype of rs2910164 was associated with an increased risk of nasopharyngeal carcinoma (NPC) (GC + GG versus CC, OR = 0.49; 95% CI: 0.35–0.69). The authors also demonstrated that passenger strand miR-146a*C in NPC is significantly increased in CC genotype, resulting in the regulation of a set of target genes [13]. The allelic variants of rs2910164 were evaluated in familial breast and ovarian cancers in BRCA1/BRCA2-negative patients in a study which suggested that the polymorphism may impact on the age of cancer onset. In fact, subjects with GC or CC genotypes developed tumors at younger age compared with individuals carrying the GG genotype [14]. However, a large study of breast cancer cases negative for disease-causing mutations or unclassified variants in BRCA1 and BRCA2 showed no associations between rs2910164 genotype and breast cancer susceptibility [15]. Another study showed also lack of association of the rs2910164 SNP with breast cancer risk in a series of BRCA1 and BRCA2 mutation carriers [16]. In a meth-analysis study, a stratified analysis by ethnicity showed that the rs2910164 polymorphism is associated with increased breast cancer risk among Europeans in a recessive model (CC versus GC + GG: OR = 1.31; 95% CI: 1.05–1.65) [17]. In another meta-analysis was shown that SNP rs2910164 is not associated with the risk of hepatocellular carcinoma [18]. Thus, the C allele of rs2910164 seems to be associated with cancer risk in a cancer type specific manner, but further studies are required to better clarify this matter. In addition, other functional studies should define the role of the polymorphism in the expression of miR-146a.

The SNP rs11614913 is located in the pre-miRNA region of miR-196a2 and has two allelic forms, T and C. Hu et al. found that in nonsmall cell lung cancer patients who were homozygous CC at SNP rs11614913, risk of death significantly increased (hazard ratio HR = 1.76; 95% CI: 1.34–2.32). The genotype rs11614913 CC was associated with a significant increase in mature hsa-mir-196a expression without changes in levels of the precursor, suggesting an enhanced processing of the pre-miRNA [19, 20]. The miR-196a2 CC genotype was also associated with reduced survival in patients with pharyngeal tumors [21] increased colorectal cancer risk [22] and increased breast cancer risk [23] compared with the TT/CT genotype. However, in breast cancer, Catucci et al. found no significant increased risk in rs11614913 CC homozygous patients [15]. A meta-analysis of 15 studies showed that individuals with the TC/CC genotypes are associated with higher cancer risk than those with the TT genotype (OR = 1.18; 95% CI: 1.03–1.34; $P < 0.001$) supporting the hypothesis that hsa-miR-196a2 rs11614913 polymorphism may contribute to cancer susceptibility [24].

The SNP rs6505162 is an A/C polymorphism located in the pre-miRNA region of miR-423. Ye et al. systematically evaluated the effects of 41 genetic variants in 26 miRNA-related genes on esophageal cancer risk. The most relevant finding was the association of rs6505162 SNP with reduced esophageal cancer risk, following an additive model (OR = 0.64; 95% CI: 0.51–0.80, $P < 0.0001$), being the C allele less represented in cases than in controls [25]. For the same polymorphism, a study that was performed on Caucasian Australian women showed that CC genotype confers a reduced risk of breast cancer development (OR = 0.50; 95% CI: 0.27–0.92) [26]. In a sample of Chinese patients with surgically resected colorectal adenocarcinoma, homozygous CC genotypes of SNP rs6505162 were significantly associated with both the overall survival (HR = 2.12; 95% CI: 1.34–3.34, $P = 0.001$) and the recurrence-free survival (HR = 1.59; 95% CI: 1.08–2.36) [27].

The SNP rs3746444, having the two alleles A and G, is located in the pre-miR region of miR-499. In a case-control study on a Chinese population, the variant G was associated with a significant increased risks of breast cancer (OR = 1.25; 95% CI: 1.02–1.51) in a dominant genetic model [23]. This association was not confirmed by the study of Catucci et al. performed on a Caucasian population [15]. In a meta-analysis study the rs3746444 polymorphism was significantly associated with breast cancer risk in Asian population, being the G allele responsible for the increased risk (OR = 1.10; 95% CI: 1.01–1.20) [28]. Moreover, the rs3746444 polymorphism was associated with a significant increased risk (OR = 1.98; 95% CI: 1.36–2.98; $P = 0.0004$) of cervical squamous cell carcinoma, following an overdominant model [29].

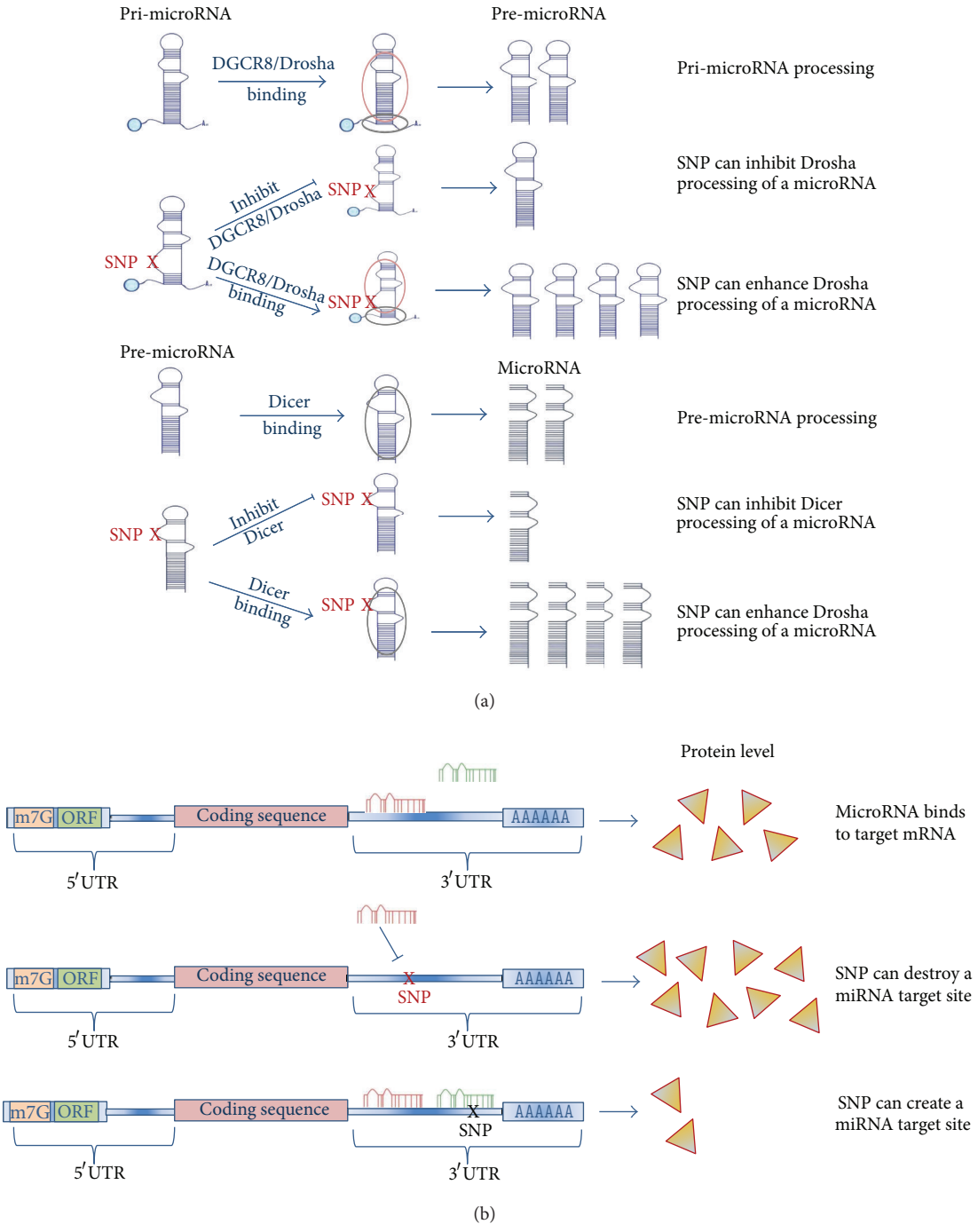

Figure 1: Schematic representation of influence of miRSNPs in microRNA processing (a) and in protein translation of microRNA target genes (b).

SNP rs4919510 is located in pre-miRNA region of miR-608 and has C and G alleles. The relevance of this polymorphism has been studied in patients with colorectal cancer (CRC). In CRC patients receiving first-line fluoropyrimidine-based chemotherapy several SNPs located in miRNA regions were genotyped, the rs4919510 resulted associated with increased risk for both recurrence and death (HR = 2.72; 95% CI: 1.38–5.33 and HR = 3.53; 95% CI: 1.42–8.73) [30]. These findings were confirmed by an independent study, in which rs4919510 was associated with altered recurrence-free survival only in patients receiving chemotherapy but not in those without chemotherapy [27]. In another study, there was no significant association found between rs4919510 and colorectal cancer risk, but the GG genotype was associated with an increased risk of death in Caucasians and with a reduced risk of death in African Americans [31]. Recently,

Table 1: List of miRSNPs evaluated in cancer susceptibility studies.

SNP category	Cancer type	SNP	Gene	OR (95% CI)	Reference
miRNA sequence					
	Any cancer	rs11614913	miR-196a2	1.18 (1.03–1.34)	[24]
	Bladder cancer	rs2910164	miR-146-a	0.80 (0.71–0.90)	[12]
	Breast cancer	rs895819	miR-27a	0.88 (0.78–0.99)	[33]
		rs2910164	miR-146-a	1.31 (1.05–1.65)	[17]
		rs11614913	miR-196a2	1.23 (1.02–1.48)	[23]
		rs6505162	miR-423	0.50 (0.27–0.92)	[26]
		rs3746444	miR-499	1.25 (1.02–1.51)	[23]
		rs3746444	miR-499	1.10 (1.01–1.20)	[28]
		rs4919510	miR-608	1.62 (1.23–2.15)	[32]
	Cervical cancer	rs3746444	miR-499	1.98 (1.36–2.89)	[29]
	Colorectal carcinoma	rs213210	miR-219-1	3.22 (1.70–6.10)	[30]
		rs6505162	miR-423	2.12 (1.34–3.34)	[27]
		rs4919510	miR-608	2.72 (1.38–5.33)	[30]
	Esophageal cancer	rs213210	miR-219-1	1.75 (1.10–2.80)	[25]
		rs6505162	miR-423	0.64 (0.51–0.80)	[25]
	Gastric cancer	rs895819	miR-27a	1.48 (1.06–2.05)	[37]
	Nasopharyngeal carcinoma	rs2910164	miR-146-a	0.49 (0.35–0.69)	[13]
	Non-small cell lung cancer	rs11614913	miR-196a2	1.76 (1.34–2.32)	[19]
	Papillary thyroid carcinoma	rs2910164	miR-146-a	1.62 (1.3–2.0)	[11]
	Renal carcinoma	rs895819	miR-27a	0.71 (0.56–0.90)	[36]
Binding site					
	Bladder cancer	1010A/G	*HOXB5*	1.48 (1.07–2.06)	[60]
	Breast cancer	rs61764370	*KRAS*	1.47 (1.05–2.06)	[39]
		rs7963551	*RAD52*	0.84 (0.75–0.95)	[50]
		rs1044129	*RYR3*	1.26 (1.03–1.54)	[59]
		rs334248	*TGFBR1*	2.2 (1.29–4.07)	[47]
	Colorectal carcinoma	rs17281995	*CD86*	2.93 (1.29–6.67)	[58]
		rs1051690	*INSR*	2.06 (1.19–3.56)	[58]
	Non-small cell lung cancer	rs61764370	*KRAS*	1.36 (1.07–1.73)	[38]
	Ovarian cancer	rs61764370	*KRAS*	1.67 (1.09–2.57)	[43]
		rs4245739	*MDM4*	5.5 (1.5–20.5)	[61]
	Small-cell lung cancer	rs3134615	*MYCL1*	2.08 (1.39–3.21)	[49]
		rs16917496	*SET8*	0.45 (0.22–0.94)	[54]
	Squamous cell carcinoma of the head and neck	rs1049253	*CASP3*	1.29 (1.07–1.56)	[56]
Biogenesis pathway					
	Breast cancer	rs11786030	*AGO2*	2.62 (1.41–4.88)	[66]
		rs2292779	*AGO2*	2.94 (1.52–5.69)	[66]
	Head and neck cancer	rs1057035	*DICER*	0.65 (0.46–0.92)	[69]
	Non-small cell lung cancer	rs11077	*XPO5*	1.77 (1.07–2.91)	[68]
	Prostate cancer	rs2740348	*GEMIN4*	0.64 (0.42–0.99)	[67]
		rs7813	*GEMIN4*	2.53 (1.07–6.28)	[67]
		rs3744741	*GEMIN4*	0.69 (0.50–0.96)	[67]
	Renal carcinoma	rs2740348	*GEMIN4*	0.67 (0.47–0.96)	[64]
		rs7813	*GEMIN4*	0.68 (0.47–0.98)	[64]
		rs910925	*GEMIN4*	1.74 (1.15–1.62)	[65]
		rs3744741	*GEMIN4*	0.39 (0.19–0.77)	[65]

rs4919510 was studied in breast cancer patients, and the G-allele was specifically associated with an increased risk of HER2-positive subtype (OR = 1.62; 95% CI: 1.23–2.15) [32].

The SNP rs895819 is located in the pre-miRNA-27a and has A and G alleles. The role of this SNP was first investigated in a German familial breast cancer study cohort, in which SNPs related to other microRNAs were also analyzed. The G allele of SNP rs895819 resulted significantly less frequent in cases than in controls, indicating a lower familial breast cancer risk for patients carrying this variant (OR = 0.88; 95% CI: 0.78–0.99) [33]. The same conclusion was not confirmed in an Italian breast cancer cohort with similar characteristics [34]. The discordance between these two studies was further investigated, and it is probably due to technical aspects concerning the genotyping method used [35]. In a case-control study on renal carcinoma, individuals carrying AG/GG genotypes of SNP rs895819 had a statistically significant lower susceptibility in the development of renal cancer (OR = 0.71; 95% CI: 0.56–0.90) than individuals with AA genotype [36]. In another case-control study which was performed to investigate the role of SNP rs895819 in gastric cancer susceptibility, subjects with the variant genotypes (AG + GG) showed an increased risk of gastric cancer relative to AA carriers (OR = 1.48; 95% CI: 1.06–2.05) [37]. Moreover, patients with AA genotypes showed higher levels of miR-27a compared with AG and GG genotypes, and the miR-27a levels resulted inversely correlated with the expression of ZBTB10, an miR-27a target gene.

The SNP rs213210 is a C/T variation in the region of miR-219-1. This polymorphisms was associated with increased risk in esophageal cancer with a dominant model (OR = 1.75; 95% CI: 1.10–2.80) [25] and increased risk of death in patients with colorectal cancer receiving first-line fluoropyrimidine-based chemotherapy (HR = 3.22; 95% CI: 1.70–6.10) [30].

3. SNPs in microRNA-Targeted 3′UTRs

SNP variants located in 3′UTRs can destroy or create miRNA binding sites influencing tumor susceptibility. In order to evaluate the importance of the SNPs in cancer susceptibility, both microRNAs and target genes should have relevance in the tumor type considered.

To establish the role in cancer susceptibility of SNPs located in the 3′UTR of miRNA-targeted genes, beyond association studies, two kinds of functional assays should be performed. First, both SNP allelic forms of the 3′UTR should be cloned downstream of a reporter gene (usually a luciferase) and in cells cotransfected with the interacting micro-RNA, and the alternative 3′UTRs forms the quantified luciferase activity should be significantly different. Second, in biological samples the levels of protein or messenger RNA of the different allelic forms should be dependent on the expression of the interacting microRNA.

Among the SNPs located in the 3′UTRs, the most studied is the rs61764370, which is located in the *KRAS* gene. It has G and T alleles and influences the binding of *KRAS* with let-7. Remarkably, both *KRAS* and let-7 are important in cancer development being *KRAS* a well-known oncogene and let-7 a microRNA that acts as tumor suppressor gene [4]. Therefore, the rs61764370 polymorphism fulfills the criteria of relevance in cancer predisposition. This polymorphism was studied for the first time by Chin et al. in nonsmall lung cancer patients [38]. These authors showed that the less common G variant of rs61764370 disrupts significantly the binding of microRNA let-7 to the 3′UTR of *KRAS*, increasing *KRAS* expression. In addition, in that study was found that subjects (smoking < 40 pack/year) with the GG + GT genotypes have an increased risk (OR = 1.36; 95% CI: 1.07–1.73; $P = 0.01$) of nonsmall lung cancer. After this first study, G variant of rs61764370 was found associated with higher risk in breast cancer [39], colorectal cancer [40], melanoma [41], oral cancers [42], and ovarian cancer [43]. Conversely, other authors did not find significant association of rs61764370 with cancer risk in colorectal cancer [44], nonsmall lung cancer [45], and ovarian cancer [46]. Thus, the relevance of rs61764370 in cancer predisposition is still debated and deserves further investigations.

The SNP rs334348 is located in the 3′UTR of *TGFBR1* gene and has A and G variants. Nicoloso et al. found that subjects with AG genotype has higher susceptibility to familiar breast cancer (OR = 2.2; 95% CI: 1.29–4.07; $P = 0.005$) [47]. Functionally, it was shown that the G allele of rs334348 is targeted with higher efficiency by miR-628-5p than the A allele. This was demonstrated by luciferase assay using the cloned alternative forms of *TGFBR1* 3′UTR and by western blot using breast cancer cell lines with different genotypes. Notably, the expression of TGFBR1 influences significantly the colorectal cancer risk [48].

The SNP rs3134615 is in the 3′-UTR of *MYCL1*, within a miR-1827 binding site, and has G and T alleles. In a case-control study, Xiong et al. showed that subjects with TG and TT genotypes have an increased risk (OR = 2.08; 95% CI: 1.39–3.21; $P = 0.0004$) for the development of small-cell lung cancer [49]. Moreover, the same authors demonstrated that the T allele significantly impairs the interaction of miR-1827 with the 3′UTR of *MYCL1*, reducing miR-1827 dependent inhibition of *MYCL1* expression.

The SNP rs7963551 is located in the 3′UTR of *RAD52* and has C and A variants. *RAD52* encodes a protein involved in homologous recombination repair. In a study performed to evaluate the relevance of rs7963551 on breast cancer susceptibility, the C allele was associated with reduced cancer risk (OR = 0.84; 95% CI: 0.75–0.95). Moreover, a reduced inhibition of *RAD52* expression of C allele, probably due to a weakened binding capacity of hsa-let-7 to 3′-UTR of *RAD52*, was demonstrated by luciferase activity assay in MCF-7 cell line [50].

The rs16917496 is a C/T variation located in the 3′UTR of *SET8* within the miR-502 binding site, having the C allele a perfect match in the seed region of miR-502. The expression of *SET8* in breast tumor tissues of patients with a CC genotype is significantly lower than in patients with TT genotype, and, importantly, the age of breast cancer onset depends on the number of C alleles, being significantly lower in C allele carriers ($P = 0.022$) [51]. This is in agreement with the role played by *SET8*, a methyltransferase that acts on p53 increasing its proapoptotic potential [52]. It was found that the *SET8* CC genotype confers also longer survival to

patients with hepatocellular carcinoma [53] and with small-cell lung cancer [54], and it is associated with a decreased risk of epithelial ovarian cancer [55].

The rs1049253 is a C/T variation located in the 3′UTR of *CASP3* gene within the binding site of miR-885-5p. *CASP3* belongs to the caspase family genes encoding key effector enzymes involved in cell apoptosis. Downregulation of caspases affects programmed cell death allowing tumor cell proliferation. A study on 7 SNPs located in the 3′UTR of caspase genes was performed to assess their possible association with squamous cell carcinoma of the head and neck. The genotypes CC/CT rs1049253 resulted associated with significantly increased cancer risk (OR = 1.29; 95% CI: 1.07–1.56), but no associations were found for the other 6 SNPs. Moreover, the rs1049253 CC genotype was associated with reduced levels of *CASP3* mRNA compared with the TT genotype, and C allele resulted in stronger down-regulation than T allele of the *CASP3* expression determined with miR-885-5p mimic transfection and luciferase assay [56].

In a study of Landi et al. [57], SNPs located in the miRNA binding sites of 3′UTR of genes relevant in the pathogenesis of colorectal cancer (CRC) were computationally tested for their ability to affect the binding of the miRNA with its target. Eight polymorphisms were further studied by case-control studies and two of them, rs17281995 and rs1051690, located in the *CD86* and *INSR* genes respectively, resulted significantly associated with CRC risk. In a subsequent paper [58], the same authors extended the study of rs17281995 and rs1051690 polymorphisms in CRC risk, analyzing an additional population (OR = 2.93; 95% CI: 1.29–6.67, for rs17281995 and OR = 2.06; 95% CI: 1.19–3.56, for rs1051690, in the pooled samples, following a codominant model).

The SNP rs1044129 is a G/A variation located in the 3′UTR of *RYR3* gene in the miR-367 binding site. *RYR3* encodes a protein that forms a calcium channel which is relevant for cell growth and migration of breast cancer cells. In a case-control study, AA genotype carriers showed a significant higher breast cancer risk (OR = 1.26, 95% CI: 1.03–1.54) than G allele carriers (GA + GG genotypes) [59]. Moreover, breast cancer patients with AA genotype had a significantly higher progression-free survival (P = 0.036) than patients carrying the G allele. The authors also demonstrated by functional assays that the A allele is more repressed by miR-367 than the G allele.

HOXB5, a member of the *HOX* gene family, has a SNP (1010A/G) in its 3′UTR in the binding site of miR-7. In a comprehensive study, Luo et al. [60] first showed the importance of *HOXB5* in the pathogenesis of bladder cancer demonstrating that inhibition of its expression decreases bladder cell proliferation and tumorigenicity. Then, the authors found that subjects carrying the G allele (AG + GG genotypes) have a higher bladder cancer risk (OR = 1.48; 95% CI: 1.07–2.06) compared with AA genotype carriers. In agreement with the above results, the authors showed by luciferase assay that the 1010A allele is repressed with higher efficiency by miR-7 than the 1010G allele.

The rs4245739 is an A/C variation located in the 3′UTR of *MDM4* in an miR-191 binding site. Overexpression of MDM4 protein promotes tumorigenesis by the negative regulation of P53 activity. In experiments performed in ovarian cancer cells, Wynendaele et al. [61] showed a stronger inhibitory effect of miR-191 on the A allele compared with C allele. Furthermore, the authors showed that patients with ER negative ovarian cancer and homozygous for the A allele have an increased risk of tumor-related death (HR = 5.5; 95% CI: 1.5–20.5).

4. SNPs in Genes Encoding microRNA Processing Proteins

Polymorphisms which influence the expression of proteins involved in miRNA biogenesis pathway may affect miRNA-mediated regulation within the cell and may contribute to the genetic variation observed in specific phenotypes [62]. Several studies focused on miRSNPs of the silencing machinery, and here we summarize this knowledge.

A Korean study [63] of 2010 evaluated *AGO1*, *AGO2*, *TNRC6A*, *TNRC6C*, *TARBP2* and *XPO5* mutations in colorectal (CRC) and gastric cancers (GC), with or without microsatellite instability. Data of this study indicate that frameshift mutations in *AGO2* and *TNRC6A* and their losses of expression are common in GCs and CRCs with microsatellite instability and suggest that these alterations may contribute to the cancer development by deregulating miRNAs.

In a study on renal cancer [64], 40 SNPs from 11 miRNA processing genes (*DROSHA*, *DGCR8*, *XPO5*, *RAN*, *DICER1*, *TARBP2*, *AGO1*, *AGO2*, *GEMIN3*, *GEMIN4*, and *HIWI*) were genotypized. Two SNPs in the *GEMIN4* gene were significantly associated with altered renal cell carcinoma risk. The variant-containing genotypes of Asn929Asp and Cys1033Arg significantly reduced risk, with odds ratios of 0.67 (95% CI: 0.47–0.96) and 0.68 (95% CI = 0.47–0.98), respectively. Haplotype analysis showed that a common haplotype of *GEMIN4* was associated with a significant reduction in the risk of renal cell carcinoma (OR = 0.66; 95% CI: 0.45–0.97).

The same research group, in a successive study [65], took a polygenic approach to evaluate the effects of 41 potentially functional SNPs in miRNAs-related genes on survival and recurrence among renal cell carcinoma (RCC) patients. In single-SNP analysis, seven SNPs were identified significantly associated with RCC survival and five SNPs with recurrence. The most significant associations were SNPs in *GEMIN4* with the variant alleles of both rs7813 and rs910925 associated with 1.74-fold (95% CI: 1.15–2.62) increased risk of death, whereas the variant allele of rs3744741 conferred a decreased risk of death (HR = 0.39; 95% CI: 0.19–0.77). Haplotypes of *DICER* and *DROSHA* were also associated with altered patient survival and recurrence.

To test the hypothesis that adverse alleles in miRNA-related genes may influence the risk for esophageal cancer, the associations between esophageal cancer risk and 41 potentially functional SNPs in 26 miRNA-related genes were assessed, in a case-control study [25]. A common haplotype of the *GEMIN4* gene was associated with a significantly reduced risk of esophageal cancer (OR = 0.65; 95% CI: 0.42–0.99).

In a recent study [66], the authors used genotype data available from a previous case-control study to investigate

association between common genetic variations in miRNA biogenesis pathway genes and breast cancer survival. They investigated the possible associations between 41 SNPs and both disease-free survival (DFS) and overall survival (OS) among 488 breast cancer patients. Two SNPs in AGO2 (rs11786030 and rs2292779) and *DICER1* rs1057035 were associated with both DFS and OS. Two SNPs in *HIWI* (rs4759659 and rs11060845), and *DGCR8* rs9606250 were associated with DFS, while *DROSHA* rs874332 and *GEMIN4* rs4968104 were associated with OS only. The most significant associations were observed in variant allele of *AGO2* rs11786030 with 2.62-fold increased risk of disease progression (95% CI: 1.41–4.88) and in minor allele homozygote of *AGO2* rs2292779 with 2.94-fold increased risk of death (95% CI: 1.52–5.69).

A recent study was performed in order to evaluate the role of miR-SNPs of *GEMIN4* in prostate cancer [67]. The high-resolution melting method was used to genotype seven polymorphisms (rs7813, rs4968104, rs3744741, rs2740348, rs1062923, rs910925, and rs910924) in the *GEMIN4* gene. Patients carrying the variant heterozygote GC genotype in the rs2740348 were at a 36% decreased risk of prostate cancer (OR = 0.64; 95% CI: 0.42–0.99). In addition, subjects carrying the homozygote TT genotype in the rs7813 had a significantly increased risk of prostate cancer (OR = 2.53; 95% CI = 1.07–6.28). Two common haplotypes were found to be associated with decreased risk of prostate cancer. In the subgroup analysis, higher risk of more severity of prostate cancer (clinical stage III and IV) was observed in individuals with the rs7813 TT genotype (OR = 2.64; 95% CI: 1.02–7.64), while lower risk of more severity of prostate cancer was observed in individuals with the rs3744741 T allele (OR = 0.69; 95% CI: 0.50–0.96).

In a study on a sample of nonsmall-cell lung cancer patients [68], three miR-SNPs in microRNA-processing machinery components were examined, and the time to recurrence (TTR) according to miR-SNP genotypes was evaluated. Significant differences in TTR were found for *XPO5* rs11077 (median TTR: 24.7 months for the AA genotype versus 73.1 months for the AC or CC genotypes; $P = 0.029$). In multivariate analyses, the *XPO5* rs11077 AA genotype (OR = 1.77; 95% CI: 1.07–2.91) emerged as independent variable influencing TTR.

In a recent case-control study on head and neck cancer (HNC) [69], three SNPs at miRNA binding sites of miRNA processing genes were genotyped (rs1057035 in 3′UTR of *DICER*, rs3803012 in 3′UTR of *RAN,* and rs10773771 in 3′UTR of *HIWI*). Although none of the SNPs was significantly associated with overall risk of HNC, rs1057035 in 3′UTR of *DICER* was associated with a significantly decreased risk of oral cancer (TC/CC versus TT, OR = 0.65; 95% CI: 0.46–0.92). Furthermore, luciferase activity assay showed that rs1057035 variant C allele led to significantly lower expression levels of DICER as compared to the T allele, which may be due to the higher inhibition of hsa-miR-574-3p on *DICER* mRNA. These findings indicated that rs1057035 located at 3′UTR of *DICER* may contribute to the risk of oral cancer by affecting the binding of miRNAs to *DICER*. Large-scale and well-designed studies are warranted to validate these findings.

A sample of patients with chemosensitive multiple myeloma intensified with autologous stem cell transplantation was studied in a longitudinal study [70]. The SNP of miRNA biogenesis pathway evaluated was the rs11077 of *XPO5*. Overall survival was significantly longer in patients with C/C or A/C variant in *XPO5* rs11077 ($P = 0.012$). A significant longer progression-free survival for this SNP ($P = 0.013$) was also found.

5. Conclusions

The study of polymorphisms, affecting miRNA-dependent pathways and involved in cancer susceptibility, is rapidly growing, and in the near future probably other SNPs will be investigated, and the SNPs mentioned in this paper will be further evaluated. The acquisition of new data in different populations has a paramount importance to establish the real contribution of each polymorphism to the cancer risk in this promising area.

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Research Article

Contribution of Large Genomic Rearrangements in Italian Lynch Syndrome Patients: Characterization of a Novel Alu-Mediated Deletion

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Lynch syndrome is associated with germ-line mutations in the DNA mismatch repair (MMR) genes, mainly *MLH1* and *MSH2*. Most of the mutations reported in these genes to date are point mutations, small deletions, and insertions. Large genomic rearrangements in the MMR genes predisposing to Lynch syndrome also occur, but the frequency varies depending on the population studied on average from 5 to 20%. The aim of this study was to examine the contribution of large rearrangements in the *MLH1* and *MSH2* genes in a well-characterised series of 63 unrelated Southern Italian Lynch syndrome patients who were negative for pathogenic point mutations in the *MLH1*, *MSH2*, and *MSH6* genes. We identified a large novel deletion in the *MSH2* gene, including exon 6 in one of the patients analysed (1.6% frequency). This deletion was confirmed and localised by long-range PCR. The breakpoints of this rearrangement were characterised by sequencing. Further analysis of the breakpoints revealed that this rearrangement was a product of Alu-mediated recombination. Our findings identified a novel Alu-mediated rearrangement within *MSH2* gene and showed that large deletions or duplications in *MLH1* and *MSH2* genes are low-frequency mutational events in Southern Italian patients with an inherited predisposition to colon cancer.

1. Introduction

Hereditary nonpolyposis colorectal cancer (HNPCC; also known as Lynch syndrome) is an autosomal dominant disorder characterised by colorectal cancer [1] that accounts for 3–5% of all colorectal cancers. Affected individuals have approximately 60–80% lifetime risk of developing colorectal cancer and women with Lynch syndrome have 54% risk of developing endometrial cancer [2]. It is associated with germ-line mutations in the DNA mismatch repair (MMR) genes, mainly *MLH1* and *MSH2* [3]. Mutations in *MSH6* [4], *PMS2* [5], and *MLH3* [6] are less common. Recently, a germ-line point mutation in *MSH3* was found to be associated with the Lynch syndrome phenotype [7]. Inactivation of the MMR complex manifests microsatellite instability (MSI), which is detected in tumour tissue [8].

The majority of mutations in the MMR genes so far identified are missense, nonsense, or small insertions/deletions [http://www.insight-group.org/mutations mutations]. Depending on the population studied, large genomic rearrangements of the MMR genes constitute various proportions of the germ-line mutations that predispose to HNPCC [9–11]. Moreover, it seems that large genomic rearrangements occur more frequently in some populations than in others [11, 12]. The relative incidence of genomic rearrangements among Lynch Syndrome families appears to vary from 5–20% [13]. A systematic study on genomic rearrangement in Lynch Syndrome showed

that *MLH1* and *MSH2* are the most frequently targeted MMR genes for this type of mutation [14]. Furthermore, molecular characterisation of the breakpoints involved in large rearrangements within *MLH1* and *MSH2* genes showed that the majority are caused by homologous recombination between Alu repeats [15–17]. These mutations are not usually detected by conventional methods of mutation analysis, such as denaturing high-performance liquid chromatography (DHPLC) and direct DNA sequencing, but they are detectable by a simple and robust technique such as the Multiplex Ligation-Probe Dependent Amplification (MLPA) [18, 19] assay.

As little is known about the frequency of large rearrangements in the *MLH1* and *MSH2* genes to Lynch syndrome in Italian population, the aim of our study was to assess the contribution of large genomic rearrangements in these two genes in a well-characterised series of 63 Southern Italian patients affected by Lynch Syndrome.

2. Materials and Methods

2.1. Patients. Sixty-three families of Italian origin, 56 families classified according to the Amsterdam criteria [20] and 7 atypical Lynch families selected according to MSI high status (MSI-H) [20], without germ-line pathogenic point mutations in the MLH1, MSH2, or MSH6 genes, were recruited from several health centres in Campania (Southern Italy).

All patients received genetic counselling and gave their written informed consent to participate in this study.

2.2. Isolation of Genomic DNA. Total genomic DNA was extracted from 4 mL peripheral blood lymphocytes using a Nucleon BACC2 Kit (Amersham Life Science) and from tumour tissues and surgical margins by standard methods [21].

2.3. Multiplex Ligation-Dependent Probe Amplification (MLPA). MLPA was performed using the SALSA MLPA P003-B1 ML H1/MSH2 kit (MRC-Holland, The Netherlands) according to the manufacturer's instructions. Fragment analysis was conducted on an ABI Prism 3130 Genetic Analyser using GeneMapper software (Applied Biosystems, Foster City, CA, USA). Migration of fragments was calculated by comparison to the GeneScan LIZ-500 size standard (Applied Biosystems, Foster City, CA, USA). Peak areas were then exported to a Microsoft spreadsheet (www.MLPA.com) and calculations were done according to the method described by Taylor and colleagues [22]. A 30–50% decrease in the peak area(s) indicated a deletion of the corresponding exon(s), while a 30–50% increase in the peak area(s) indicated a duplication of the corresponding exon(s). MLPA results were confirmed in at least two independent experiments.

2.4. DNA Amplification and Microsatellite Analysis. The MSI status was confirmed with a fluorescent multiplex system [23] comprising six mononucleotide repeats (BAT-25, BAT-26, BAT-40, NR-21, NR-24, and TGFβRII) and four dinucleotide repeats (D2S123, D5S346, D17S250, and D18S58). 20 ng of DNA extracted by tumor tissue and peripheral blood lymphocytes were amplified in 25 μL reaction volume using the CC-MSI Kit (Ab Analitica, Padova, Italy), in according to manufacture instructions. The PCR products were analysed by capillary electrophoresis analysis using an ABI Prism 3130 Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

2.5. RNA Analysis of MSH2 Gene. RNA was extracted from 4 mL peripheral blood lymphocytes using a Trizol reagent by standard methods (Quiagen). cDNA was synthesised using SuperScript II RT (Invitrogen by Life Technologies) and amplified with primers that produced a 598-bp fragment (2cFP 5′-GGCTCTCCTCATCCAGATTG and 2cRP 5′-AAGATCTGGGAATCGACGAA) spanning exons 4–7 of the messenger RNA. The PCR products were analysed on a 2% agarose gel and visualised by ethidium bromide staining.

2.6. Long-Range Polymerase Chain Reaction and Breakpoint Analysis. 500 ng of genomic DNA was amplified in a 50 μL-reaction volume using 2.75 mM Mg^{2+}, 500 μM of each dNTP, 2 U of Expand Long Template PCR System (Expand Long Template Buffer 2; Roche Diagnostics), and 300 nM of each primer. Primers were designed between exon 5 and intron 7 of the *MSH2* gene. This region was amplified in four PCR fragments. The same forward oligonucleotide (5FP) was used in each reaction with a different reverse oligonucleotide, each approximately 1000 bp apart (Table 1). Cycling conditions were as follows: 94°C for 2 min, followed by 10 cycles consisting of 94°C for 10 sec, 60°C for 30 sec (−0.5°C/cycle) and 68°C for 15 min, followed by 25 cycles consisting of 94°C for 15 sec, 57°C for 30 sec, and 68°C for 15 min (+20 sec/cycle), and finishing with one cycle at 68°C for 7 min.

All oligonucleotides were designed using Primer3 Software (http://frodo.wi.mit.edu/primer3/) and checked using the Basic Local Alignment Search Tool program (BLAST, http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.7. Sequencing Analysis. The PCR products were sequenced in both the forward and reverse directions using an ABI Prism 3100 Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

2.8. In Silico Analysis. The nucleotide sequences of the genomic *MSH2* region (NG_007110.1) were analysed with the RepeatMasker program (http://repeatmasker.org/) using the default settings. Sequence comparisons in RepeatMasker were performed by the program cross_match [24].

3. Results

3.1. Detection of Large Genomic Rearrangements in the MSH2 and MLH1 Genes by MLPA. MLPA analysis on 63 unrelated patients identified a deletion in the *MSH2* gene in one patient only (1.6%) (Figure 1). This deletion removed exon 6, which is located between the small intron 5 and the large intron 6. The exon 6 deletion was confirmed at the RNA level by

Table 1: Primer sequences used for long-range PCR to characterise the breakpoints of the MSH2 exon 6 deletion.

Primer	Sequence	Nucleotide position (NG_007110.1)	Amplicon size (bp)
5FP	GGATATTGCAGCAGTCAGAGCCC	11258–11280	
7RP	AGAGTGAGTCACCACCACCAACT	26890–26913	15657 bp
6RPl	AGCTTTTCTGGAGGCCATAGGCA	24694–24717	13459 bp
6RPi	AGTCTGGTCCAAGGATCACCAGCA	23620–23644	12386 bp
6RPh	TCGTCGGTGGAAGAGGTGGCT	22565–22586	11328 bp
6RPg	AGCCCATGAAGAGAGCTGACACC	21580–21603	10345 bp

RT/PCR sequencing of a fragment with a lower molecular weight. The deletion was identified in a 39-year-old man with a family history of colorectal cancer, who had developed a tubulovillous adenoma with small fragments of mucinous adenocarcinoma in the rectum, approximately 75 cm from the anus. The same deletion was also detected in his 33-year-old brother. Although the brother was asymptomatic, endoscopy revealed an adenocarcinoma located proximal to the hepatic flexure (Figure 2).

3.2. Microsatellite Analysis. MSI analysis was performed on DNA extracted from tumour tissues (adenocarcinoma), and surgical margins of both patients (the proband and his brother) carrying the *MSH2* exon 6 deletion. Both patients were found to have an MSI-H status, with instability at all markers analysed (data not shown).

3.3. Breakpoint Characterisation of the MSH2 Exon 6 Deletion. The breakpoints of the exon 6 deletion within the *MSH2* gene were characterised by analysing the intragenic regions between exon 5 and exon 7. This region was amplified using region-specific oligonucleotides, as described in the Materials and Methods section. One forward primer located in exon 5, and different reverse primers starting in exon 7 were used. Abnormal fragment products of 3804, 2731, 1673 and 690 bp were amplified from the patient's DNA but not from the DNA of the healthy control using the primer pairs 5FP/6RPl, 5FP/6RPi, 5FP/6RPh, and 5FP/6RPg, respectively. No amplification products were obtained using the primer pair 5FP/7 RP.

As shown in Figure 3, sequence analysis of the 690-bp amplification product obtained using the primer pair 5FP/6RPg revealed the loss of a 9655-bp genomic region. The 5′ breakpoint is located in intron 5, in a strech of 11 nucleotides located 1,535–1,525 nt before the first nucleotide of exon 6. The 3′ breakpoint is located in intron 6, in an identical sequence of 11 nucleotides located 5,325–5,315 nt before the first nucleotide of exon 7. The exact breakpoints could not be ascertained because of the presence of an identical 11-bp sequences at both ends. This deletion c.942+(346–356)_1077-(5323–5313)del, alternatively NC_000002.11:g.47641903_47651558del, is named in accordance with the mutation nomenclature instructions provided by the HGVS (http://www.hgvs.org/); it creates a premature stop codon and the formation of a truncated protein.

3.4. In Silico Analysis. Using the RepeatMasker program, the 5′ and 3′ breakpoints of the 9655-bp deletion were found to lie within the 26-bp core sequence of two Alu elements, which share 96% homology and differ by only one nucleotide. Both Alu elements belong to the AluSx subfamily and were 269 bp and 310 bp, respectively. Homology analysis of the AluSx sequences included in the deletion was performed using BLAST analysis (Figure 4).

The entire *MSH2* gene was also analysed by RepeatMasker program, as already described in the literature [25], to verify the presence of repeat sequences. In this study, a total of 190 repeat sequences, including 106 Alu-type SINE sequences, 19 L1-type LINE sequences, 12 simple repeat sequences, and 12 LTR sequences were identified, and their positions on the gene defined. Of these, 32 Alu-type SINE sequences, one L1-type LINE sequence, one LTR, and three simple repeat sequences were located in the genomic region between exons 5 and 7.

4. Discussion

The Lynch syndrome, caused primarily by germ-line point mutations within MMR genes, is also associated with large rearrangements that account for 5–20% of all mutations. Here, we report the results of our screening for large rearrangements in the *MLH1* and *MSH2* genes in a cohort of 63 Southern Italian patients who were negative for pathogenic point mutations in the *MLH1*, *MSH2*, and *MSH6* genes. We identified one large rearrangement in the *MSH2* gene and none in the *MLH1* gene. Therefore, large rearrangements in the *MLH1* and *MSH2* genes occur at a low frequency in our patient cohort (1.6%).

The rearrangement in *MSH2* identified in this study caused a large deletion that removed exon 6 and was detected in two patients from the same family who met the Amsterdam-1 criteria. The two affected brothers presented colorectal cancer with early-onset, before 40 years of age. Other family members were also affected (not tested in this study) and presented with the same phenotype (Figure 2). DNA extracted from the tumour tissues of the two patients showed an MSI-H status, with instability at all markers analysed. The novel deletion is 9,655 bp long and extends from a region 346 bp downstream of exon 5 to 5323 bp upstream of exon 7. The exact breakpoints could not be ascertained because of the presence of identical 11-bp sequences at both ends; in fact using the RepeatMasker program, the breakpoints of this deletion were found to lie

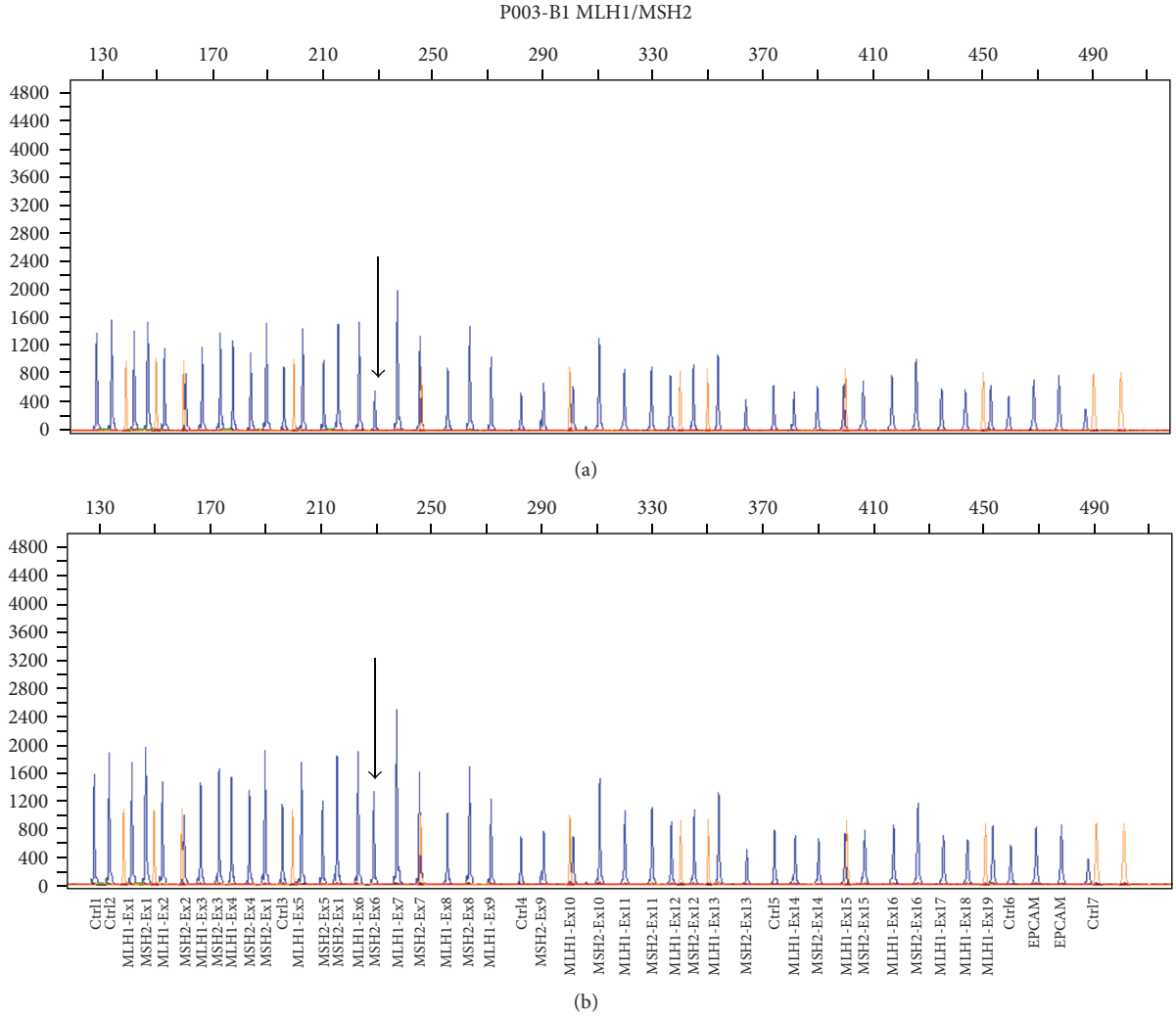

Figure 1: MLPA analysis reveals a candidate genomic rearrangement in the MSH2 gene. (a)The electropherogram of the DNA patient: the arrow shows half the level of amplification of exon 6 in the carrier subject. (b) The electropherogram of the DNA healthy control: the arrow shows normal level of amplification of exon 6.

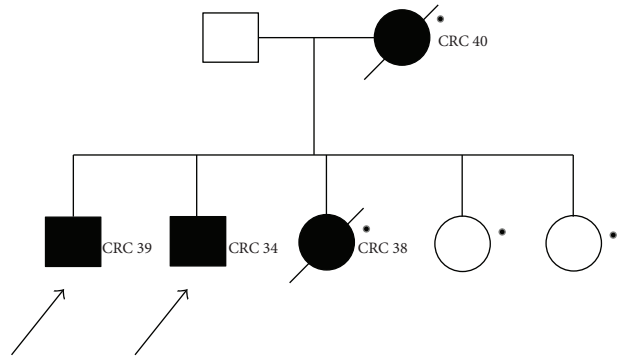

Figure 2: Family pedigree of the patient with the large MSH2 gene deletion. Symbols and abbreviations used are denoted as follows. Arrows: analysed members of family; black symbol: colorectal cancer; CRC, colorectal cancer. Number next to diagnosis denote age at oneset; •: not detected.

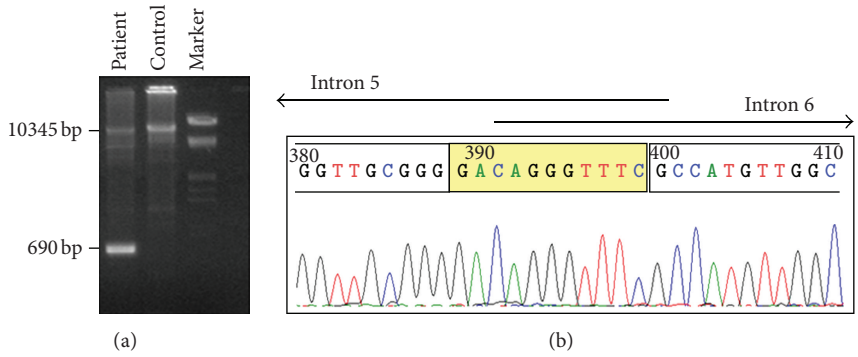

Figure 3: Confirmation and characterisation of the MSH2 exon 6 deletion. (a) Agarose gel electrophoresis (1.5%) of the long-range PCR product obtained using the forward primer located in exon 5 (5FP) and the reverse primers located in intron 6 (6RPg) (as described in the text); DNA Molecular Weight Marker III (Roche) used. An abnormal 690 bp fragment was obtained for our patient. (b) Sequence analysis of the truncated 690-bp PCR amplicon reveals the loss of a 9,655-bp genomic region. The breakpoints highlighted in yellow are located in a strech of 11 nucleotides common to both introns 5 and 6.

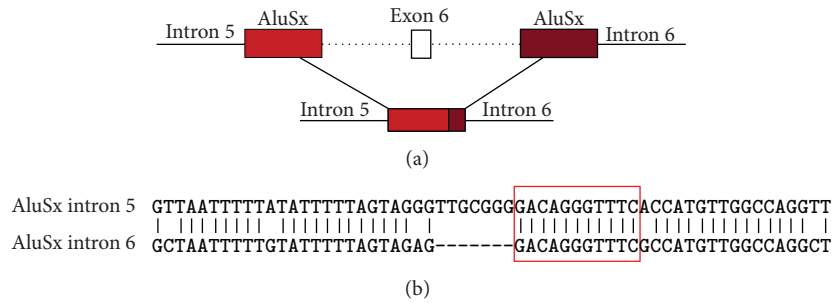

Figure 4: Detailed characteristics of Alu-mediated MSH2 exon 6 deletion. (a) Scheme of the MSH2 deletion showing that the 9655-bp deletion is located between two AluSX elements in introns 5 and 6. (b) Alignment of the two AluSX elements reveals a core 11-bp sequence identical in both introns at the breakpoint.

within the 26-bp core of two AluSx sequences that share 96% homology. As these two AluSx sequences were found to differ by only one nucleotide, it is possible that recombination could occur at this sequence. Therefore, we speculated that the *MSH2* rearrangement is most likely an Alu-Alu homologous recombination event that deletes approximately 9.5 kb of the *MSH2* genomic region encompassing exon 6.

The complete deletion of exon 6 has been previously reported to cause Lynch syndrome in a Dutch family [26], however the deletion was classified as resulting from nonhomologous recombination, as the breakpoints did not fall in Alu sequences. The breakpoint characterised in this study therefore demonstrates that we have identified a novel deletion.

The *MSH2* and *MLH1* genes are known to have a high density of Alu sequences, 34% and 21%, respectively, several large rearrangements in this gene have been reported [16, 27]. However, given the high frequency with which these repetitive sequences occur within these two genes, we would expect the overall incidence of large rearrangements in our cohort to be much higher than that identified. Therefore, it is reasonable to hypothesise that Alu-mediated homologous recombination could also cause intragenic rearrangements, such as translocations or inversions, that are not always detectable with the MLPA assay used in this study. MLPA is used for detecting copy number changes in genomic DNA and can only detect large deletions or duplications. Inability to detect intragenic rearrangements could in part explain the low frequency of these molecular alterations in our cohort. Moreover, it is noteworthy that an exceptionally low frequency of large rearrangements in the *MLH1* and *MSH2* genes (<1.5%) was also reported in a study of the Spanish population [11]; indeed, due to historical inheritage Spaniards share a common genetic pool with the Southern Italian population. In contrast, other studies performed especially on populations of Northern-Europe (including Northern Italy population) have reported an increasingly higher frequency of large rearrangements in these two genes [28, 29], with a recent study of Slovak HNPCC [12] reporting a frequency of 25%. Moreover, differences in the frequency of large rearrangements are also seen in other Alu-rich genes that are responsible for hereditary diseases, such as *BRCA1,* and *BRCA2, STK11*, depending on the population analysed [30, 31]. Therefore, based on these informations the Alu

sequences may be regarded as passive elements that serve as favourable substrates for recombination and the molecular mechanism that promotes recombination events remains to be clarified.

Beyond possible explanations about the low frequency of large rearrangements in our population, it should be highlighted that the majority of patients with Lynch syndrome tested in this study do not have a mutation in the *MMR* genes most frequently mutated. It is also important to emphasize that our families were selected on the basis of the Amsterdam clinical criteria and MSI-H, thus there is good evidence that all affected have a strong genetic component to early development of cancer. We therefore suggest that some undiscovered genetic mechanism in Lynch syndrome patients is yet to be investigated. Recently, it has been shown that unclassified genetic variants in *MMR* genes can behave as low-risk alleles that contribute to the risk of colon cancer in Lynch syndrome families when interacting together or with other low-risk alleles in other *MMR* genes [7, 32]. Furthermore, it is also possible that the existence of other as yet undiscovered genes may confer susceptibility to colon cancer in Lynch syndrome families. The *EPCAM* gene in addition to *MMR* genes has already been associated HNPCC phenotype [33] as well as *MYH* in addition to *APC* gene has been associated FAP phenotype [34]. Recently, association studies have identified a number of loci that appear confer more increases in colon cancer risk [35, 36]. Further studies are needed to better identify the underlying genetic risk factors associated with disease in these families.

5. Conclusions

This paper is the first significant study on contribution of large *MLH1* and *MSH2* genomic rearrangements in Southern Italian Lynch syndrome patients, negative for point mutation in *MMR* genes. Our results enlarge the spectrum of large rearrangements in *MSH2* genes and at the same time indicate that these genomic rearrangements seem to be a less frequent mutational event in our population. Nonetheless, we believe that the detection of large rearrangements in the *MLH1* and *MSH2* genes should be included in the routine testing for Lynch syndrome, especially considering the simplicity of the MLPA assay.

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Research Article

History, Pathogenesis, and Management of Familial Gastric Cancer: Original Study of John XXIII's Family

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Background. Hereditary diffuse gastric cancer is associated with the E-cadherin germline mutations, but genetic determinants have not been identified for familial intestinal gastric carcinoma. The guidelines for hereditary diffuse gastric cancer are clearly established; however, there are no defined recommendations for the management of familial intestinal gastric carcinoma. *Methods*. In this study we describe Pope John XXIII's pedigree that harboured gastric cancer as well as six other family members. Family history was analysed according to the International Gastric Cancer Linkage Consortium criteria, and gastric tumours were classified in accord with the last Japanese guidelines. *Results*. Seven out of 109 members in this pedigree harboured gastric cancer, affecting two consecutive generations. John XXIII's clinical tumour (cTN) was classified as cT4bN3a (IV stage). In two other cases, gastric carcinomas were classified as intestinal histotype and staged as pT1bN0 and pT2N2, respectively. *Conclusions*. Pope John XXIII's family presents a strong aggregation for gastric cancer affecting almost seven members; it spreads through two consecutive generations. In absence of defined genetic causes and considering the increased risk of gastric cancer's development in these families, as well as the high mortality rates and advanced stages, we propose an intensive surveillance protocol for asymptomatic members.

1. Introduction

About 80–90% of gastric carcinomas develop in a sporadic setting, the remaining 10% to 20% show familial cluster, and approximately only 1–3% have a clear inherited genetic conditioning [1–4]. In literature there are many reports of familial gastric cancer (FGC) with no evidence of cancer in other organs, encompassing both hereditary forms and GC clustering in families without determinant genetic susceptibility for the disease [1, 5–7].

E-cadherin gene (*CDH1*) mutations were identified as the causal event underlying the hereditary diffuse gastric cancer (HDGC) syndrome [8]. The guidelines for the management of the HDGC familial members were established by the International Gastric Cancer Linkage Consortium (IGCLC) in 1999 [2] and updated in 2010 [3]. *TP53* or mismatch repair gene (MMR) germline mutations account, respectively, for Li-Fraumeni and Lynch syndromes and, in these settings, gastric carcinoma, may develop in association with neoplastic diseases in other organs [4, 9–11].

Though the guidelines for families' management with HDGC are clearly established [3], there are no specific recommendations for families' management with other FGC's types, namely, familial intestinal gastric cancer (FIGC).

Herein we report Pope John XXIII's pedigree, displaying a clear excess of family members harbouring GC, with intestinal histotype and without cancer evidence in other organs. Furthermore, we suggest a surveillance and management for living kindred, to minimize the cancer risk in this family.

2. Methods

2.1. Familial History. Data on the family history were collected by direct interview of living members and consulting historical documents, obtained from John XXIII's personal archives. Briefly, the closest relatives were asked to report the total number of relatives in John XXIII's family (Pope John identified the proband), their ages, and their living status and the members harbouring gastric tumour, age at onset of disease, death date, and cancers in other organs. Familial aggregation was investigated with particular reference to the IGCLC criteria [2, 3]. In particular, for FIGC definition, we considered these criteria: (a) at least three relatives should have intestinal GC and one of them should be a first degree relative of the other two; (b) at least two successive generations should be affected; (c) in one of the relatives, GC should be diagnosed before the age of 50 [2].

2.2. Clinicopathological Data. Clinicopathological information were available for three members affected by primary gastric carcinoma, as illustrated in Figure 1(a) (cases IV-15, V-31, and V-32). For these cases, information about diagnosis, surgical procedure, histopathological examination, and survival were available. Regarding the proband (John XXIII), clinicopathological information were collected by consulting historical documents obtained from John XXIII's museum (Ca' Maitino museum, Sotto il Monte Giovanni XXIII, Bergamo, Italy).

Informed consent was obtained from all subjects included in this study and approved by hospital's ethics committee.

3. Results

3.1. Pedigree Analysis. Figures 1(a) and 1(b) represent the complete Roncalli family. In total, 109 members were identified, belonging to six generations. There were 66 males (60.6%) and 43 females (39.4%). Seven members were affected by gastric carcinoma; two out of six consecutive generations (IV and V) were involved. One single case of sporadic bladder cancer was identified (V-29). The GC overall frequency in this family was rather high (7/109), also considering that only two generations (IV and V) were affected. The generation IV showed the highest frequency for GC aggregation (5/41), decreasing to two GC cases in the next generation (V). So far, the last explored generation (VI) is cancer-free. Among GC patients there were four males (57.1%) and three females (42.9%); the overall mean age at onset was 75.8 years, 78.2 for males and 72.6 years for females, respectively. The youngest and the oldest ages at onset were 65 and 87 years, respectively. GC mortality rate in this family was rather high, with six of seven patients having died from causes related to tumour metastasis.

3.2. The Clinical History of John XXIII (Case IV-15). Pope John XXIII was born in Angelo Giuseppe Roncalli at Sotto il Monte (Bergamo) in Italy, on 25 November 1881. He was the fourth in a family of 13 children (Figures 1(a) and 1(b)). On August 1904 he was ordained as priest in Rome, and in 1925 Pope Pius XI named him apostolic visitator in Bulgaria, raising him to the episcopate. In 1953 he was appointed cardinal of Venice, finally at Pope Pius XII's death, Angelo Roncalli was elected Roman Pontiff on 28, October 1958, taking the name John XXIII (Figure 2). During that time, in October 1962, John XXIII convoked the Ecumenical Vatican Council II.

The clinical history began in September 1962. Firstly, he complained dyspepsia, sporadic episodes of vomiting, and weight loss (about 5 kg). X-ray examination revealed a distal gastric tumour narrowing the antrum and the angulus with pylorus substenosis and wall ulceration. Main symptoms referred by the Pope are described in detail in Table 1.

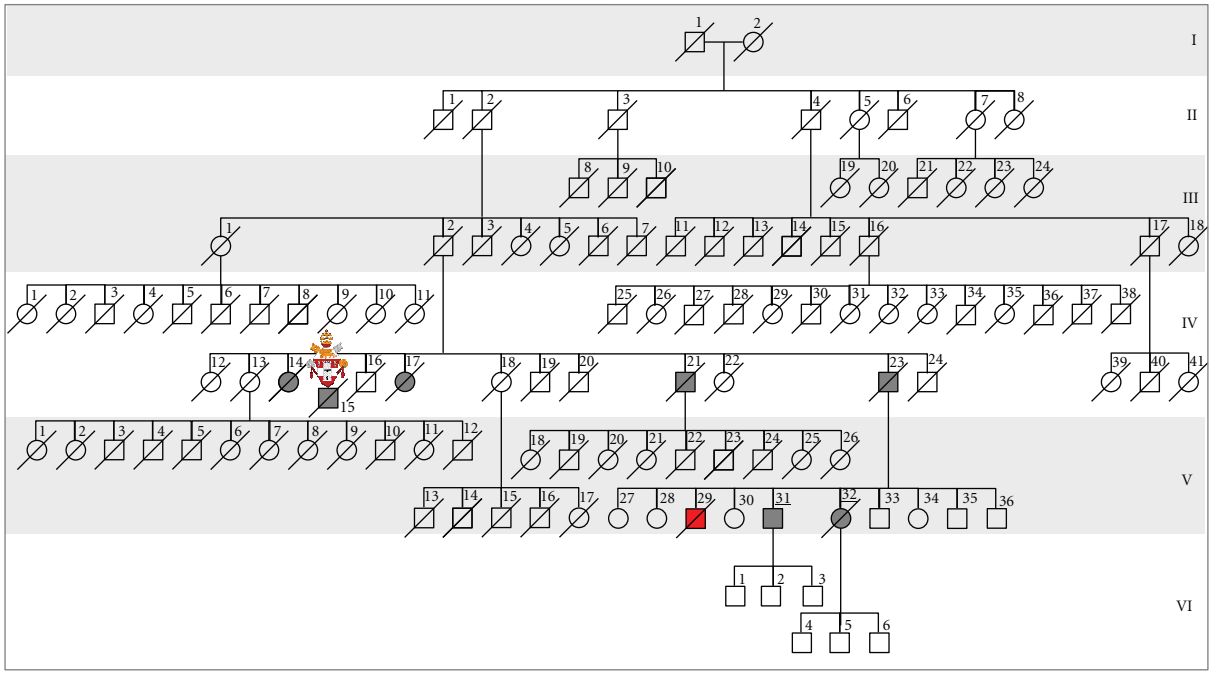
The papal physician, namely *archiatre*, convoked three eminent Italian surgeons, that visited the Pope in the papal apartments and described a palpable mass in right hypochondrium with abdominal ascites; considering the aged patient, the obesity, and other comorbidities, collegially they defined the tumour as inoperable deciding for a conservative/palliative approach. In particular, a surgeon assessed that the mortality risk for an extended gastrectomy was earnest high and whenever a radical intent was possible, the long survival's probability was very low. The conservative treatments were routinely blood and plasma transfusions, gastric mucosa extract (Opogastrina), cyclophosphamide (Endoxan), antianemic agent (Hepavis), and procoagulant drugs.

Considering these clinical reports, we could asses that the gastric tumour staging was cT4bN2 (IV stage) with intestinal histotype, because of later age at onset and slow tumour progression. However, histopathological confirmation was not available.

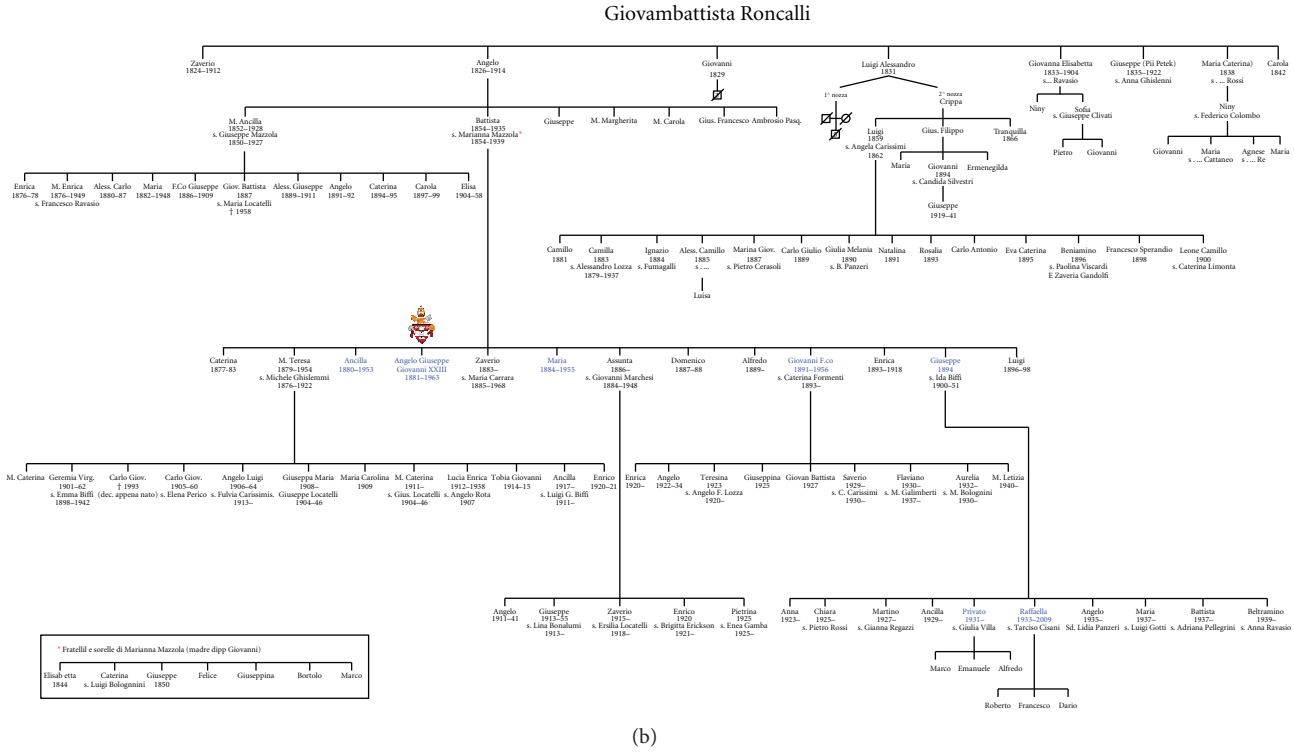
Pope John XXIII died in Vatican City in the evening of June 3, 1963, from peritonitis due to gastric carcinoma perforation. John XXIII's body was treated with chemical agents (fomaldheyde) to prevent the postmortem corruption; about 5 liters of abdominal ascites were drained.

3.3. Case V-31. Male, 79 years, was admitted at Bergamo's hospital (Italy) after incidental discovery at endoscopy of a suspicious gastric lesion; the histopathological examination of biopsies diagnosed an adenocarcinoma. There was no metastasis' evidence in other organs. The patient suffered from colon diverticular disease, abdominal aortic aneurism (treated with endovascular stent), hypertension, and prostatic hypertrophy. The patient was submitted to total gastrectomy and the pathological examination described gastric adenocarcinoma (intestinal histotype), G2 grading, with invasion of the submucosa, pT1bN0 staging. The patient is alive and well, with no evidence of local relapses or distant metastases.

3.4. Case V-32. Female, 74 years, referred vomits, nausea, diarrhoea, and body weight loss (about 15 kg). At endoscopy an infiltrative tumour was identified, causing stenosis and extending to the duodenum. The patient was submitted to subtotal gastrectomy with gastrojeiunostomy (Roux reconstruction). Due to a postoperative complication, the patient was reoperated and a total gastrectomy was performed. The pathological examination revealed gastric adenocarcinoma



(a)



(b)

FIGURE 1: (a) Schematic pedigree of John XXIII's family with seven cases affected by primary gastric carcinoma (generations IV and V). Clinicopathological information were available for cases marked with underline numbers; (b) Roncalli's original pedigree, firstly described in 1968. The bold characters indicated members affected by primary gastric tumours; the proband was indicated with the papal shield.

Figure 2: Coronation day, November 1958. Pope John XXIII with pontiff vestments wearing the papal tiara and "fanon" that defines the supreme authority as Roman Pontiff of the Catholic Church.

(intestinal histotype), G3 grading, with venous and perineural invasion. The tumour invaded the muscle layer and nodal metastases were identified in 7 out of 23 perigastric lymph nodes (pTNM stage was pT2N3a). The patient was submitted to adjuvant chemotherapy and died two years after surgery, with massive peritoneal carcinomatosis and hepatic metastases.

4. Management and Endoscopic Surveillance

4.1. Clinical Setting. A familial history as the one herein described raises several relevant issues regarding management and clinical surveillance of the asymptomatic familial members. This family fulfils the criteria for FIGC, according to the IGCLC definitions [2]. As such, this family does not qualify for the screening of E-cadherin gene (*CDH1*) germline mutations which should be offered to families with HDGC [3] and early onset GC (diffuse histotype) [12]. Moreover, the pedigree analysis excluded the possibility of Li-Fraumeni or Lynch syndromes, such as *TP53* or MMR genes' screening for a germline mutation that was not performed [4]. However, the familial members are at increased risk of GC development and management's strategy and clinical surveillance is mandatory in this family in order to reduce morbidity and mortality.

4.2. Endoscopic Surveillance. Based on the guidelines recently proposed by Kluijt and collaborators [13], we developed a protocol surveillance for asymptomatic members in this novel pedigree (Figure 3). Specifically, these guidelines recommended gastroduodenoscopy at age of 40 years (or at an age 5 years younger than youngest diagnosis in a family) with *Helicobacter pylori* testing and eradication. Attention should be given also to diet habits, namely, in GC high incidence areas and in cases with familial aggregation, based on the available evidence that indicates that specific foods, such as high consumption of grilled red meat and meat sauce, increase the risk of familial GC development [14].

Accordingly, for the family herein reported, we recommend a multidisciplinary approach with genetic counselling (Figure 3). Taking into consideration the age at onset and gender of affected kindred, as well as the GC high frequency, we suggest a periodic endoscopic surveillance, beginning at 60 years, even in the absence of symptoms. The optimal endoscopic interval is an important parameter to define. A Japanese study analysed the association between the interval of upper gastrointestinal endoscopies and the GC stage at diagnosis in patients from a GC high prevalence and in families with GC clustering [15]. These authors verified that the risk was not increased in patients in the 2- or 3-year interval group, whereas it was increased in the 4- or 5-year interval groups. In familial cases, the authors observed that in patients with a GC familial history, the risk of a GC higher stage at diagnosis was greater in patients who had a 3-year interval between endoscopies than in those with a 1-year interval and probably higher than in those with a 2-year interval. Similarly, these authors confirmed that the age of 60 years for the first endoscopy represents a valid age cut-off, particularly in families clustering for GC with abundance of intestinal histotype [16]. Other studies confirmed the utility of yearly endoscopy as the optimal interval also in other Eastern populations [15].

Thus, we suggested for this family an endoscopic yearly periodic interval. Moreover, medical examination and detailed interviews should be performed before the endoscopic procedures. Endoscopy should be performed using a white light high definition endoscope in a dedicated session with at least 30 min allocated to allow a careful inspection of the mucosa on inflation and deflation, and to allow time for multiple biopsies to be taken. Use of mucolytics such as acetylcysteine may be helpful to obtain good views [3]. Further, chromoendoscopy constitutes also an option [17]. Besides random or geographically targeted biopsies, all suspicious lesions should be biopsied [18].

5. Discussion

In 1964, Jones cited in literature a pedigree with FGC aggregation [7], corresponding to two families collected by Paulsen in 1924; in one of these families, the father, the mother and six children harboured gastric carcinoma; in the other family, the mother, and five children were affected. In 1938, Napoleon Bonaparte's family was reported [5], in which several members were affected by assured (Napoleon and his father) or suspicious GC (the grandfather, one brother, and four sisters). In 1958, Graham and Lilienfeld [6] performed genetic studies and statistical analysis of cancer developing in mono- and dizygotic twins; they found that in some specific sites, such as the stomach, if GC develops in monozygotic twin, there is an increased risk for the GC development in the other twin. In 1964, Jones identified a Maori family with a high frequency of GC; in a pedigree with 98 members, 28 were affected by primary gastric carcinoma and, within a period of 30 years, over 25 subjects died from this disease [7]. GC with familial cluster, in absence of other tumours, led

Table 1: Clinicopathological approach to John XXIII's gastric illness (Vatican City 1962-1963). As shown in this table, we considered four clinical phases.

Features	September-October 1962 Phase 1	November-December 1962 Phase 2	January–April 1963 Phase 3	May-June 1963 Phase 4
Clinical symptoms/signs	Dyspepsia, vomits, weight loss (5 kg in 4 years)	Epigastric pain, palpable mass in right hypocondrium, anemia, severe postprandial pain, nocturnal epigastric pain, insomnia, acute haemorrhage, severe anemia	Persistent epigastric pain, anorexia	Chronic epigastric pain with frequent exacerbations, multiple episodes of vomits and bleeding, melenas, strong widespread pain, anemia
Examinations/procedures	X-ray: tumour narrowing the antral region of the stomach, pyloric stenosis Ulceration	Blood and plasma transfusions, B12 vitamin, batroxobine cyclophosphamide, bicarbonate	Clinical followup	Ascites (5 litres), blood transfusion
Diagnosis/pathogenesis/evolutions	Family history	Advanced gastric cancer, cT4bN2 (IV stage), Intestinal histotype?	Unresectable gastric carcinoma	Tumour perforation, peritonitis, fever, coma, death

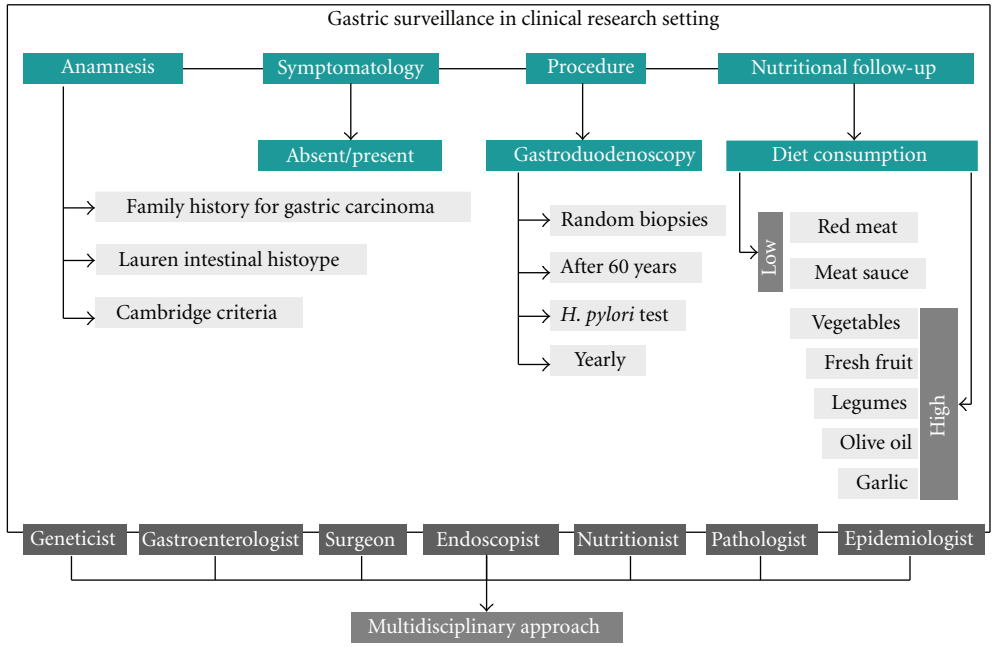


Figure 3: The proposed flow chart is suggested for the gastric surveillance in asymptomatic members recorded in this pedigree and in cases with familial intestinal gastric cancer. Some indications, such as the age for the first gastroendoscopy, are specific for this pedigree.

to the search for genetic or environmental risk factors that are associated with familial GC development's risk. In 1998, Guilford and collaborators identified, for the first time, that E-cadherin gene (*CDH1*) germline mutations constitute the genetic cause of HDGC [8]. It is now known that HDGC penetrance is about >80% [3].

Several studies showed that a familial history of GC is a risk factor for the development of the disease [19–26]. Having a first-degree relative with GC is a risk factor for GC development with odds ratio (OR) varying 2 to 10 according to the geographic region and ethnicity [27]. A large study from Turkey conferred an OR 10.1 for GC patients' siblings; nevertheless the results were not adjusted for environmental factors [28]. However, when this adjustment for environmental factors was done, it did not alter the risk. Interestingly, the Lauren GC intestinal histotype was more strongly associated with the GC familial history than the diffuse histotype [18, 23, 29].

A positive family history is considered a strong risk factor for GC development. Except for HDGC, the molecular basis for the familial aggregation is largely unknown [27].

It is believed that this GC familial cluster is due to a genetic susceptibility, shared environmental or lifestyle factors, or a combination of these in different populations. Current data shows a GC increased risk for relatives of GC patients and, in the other hand, an increased prevalence of *Helicobacter pylori* infection and premalignant lesions. There are no studies aimed to assess if the premalignant lesions of GC patient's relatives progress more rapidly through the carcinogenic cascade to GC than premalignant lesions in matched controls of general population [30]. However, so far it was not possible to identify a specific genetic cause for FIGC [1, 29]. New families with FIGC constitute nature's models that, in the future, may lead to the identification of genetic cause(s) and determinant environmental risk factors for this syndrome. Currently, it is recognized that patients at increased risk for GC due to ethnic background or familial history may benefit from surveillance [31]. Accordingly, GC familial history should be taken into account in the followup of precancerous conditions and lesions of the stomach. The Dutch working group on HGC has formulated guidelines for various aspects of medical management for families and individuals at high risk of GC developing, including criteria for referral, classification, diagnostics, and periodic gastric surveillance [13]. We took into consideration all these recommendations for the multidisciplinary protocol's design and for the asymptomatic members' surveillance of the family herein reported.

Detailed pedigrees, constructed with at least three generations, can provide important information for this purpose.

In the present study we described the GC history of Pope John XXIII and his family that was firstly recorded in 1968 (Figure 1) (Capovilla, Letters to family (1901–1962)). In this pedigree seven stomach cancer's cases in two consecutive generations were identified. By clinical history's evaluation and historical documents' exploration, it was concluded that Pope John XXIII died from a perforated GC staged at least as cT4bN3a. Perforation is a rare gastric carcinoma's complication, occurring in less than 1% of GC cases (Figure 4). In most cases, the tumour invades the serosa and displays metastatic lymph nodes in second level. The process of gastric wall perforation is sustained by infectious and ischemic factors due to the tumour neovascularisation which result in the shedding of the neoplastic tissue [32]. In this family we observed that GC appeared only in fourth and in fifth generations (XIX-XX centuries), with the highest

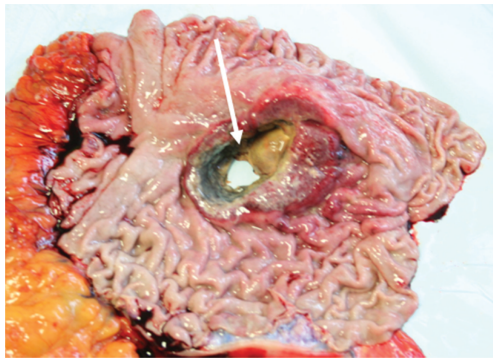


Figure 4: Representative sample of perforated gastric tumour (personal archive); arrow indicates the depth perforation.

frequency in the fourth generation. Most probably, along a time frame of about one century, this family was exposed to the same risk factors, such as environmental agents and diet habits. The putative role of genetic susceptibility and/or epigenetic changes can not be excluded.

6. Conclusions

Within familial cases, FIGC is a well recognized disease though its pathogenesis has not been fully elucidated yet. The identification of families fulfilling the criteria for FIGC requires a careful surveillance for asymptomatic members in these families. In this study we report Pope John XXIII's family, a historical family with a GC high frequency, displaying the features of intestinal carcinoma. In absence of elected genetic screening, such as searching for E-cadherin germline mutations, we proposed a pedigree-specific surveillance in asymptomatic kindred in accordance with recent guidelines. Instead, in truncating *CDH1* germline mutation carriers, prophylactic total gastrectomy represents the only life saving treatment.

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Conflict of Interests

The authors declare that they have no conflict of interests.

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