THE CHILEAN BLUE MUSSEL HAS AN INDEPENDENT CONTAGIOUS CANCER LINEAGE

A Senior Honors Thesis Presented to

the Faculty of the Department of Biology & Biochemistry

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In Partial Fulfillment

of the Requirements for the Degree

Bachelor of Science

By

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Abstract

I used the Chilean blue mussel, *Mytilus chilensis,* to identify if the leukemia-like disease in this species is a conventional cancer or a transmissible cancer, as observed in other bivalves. To test this, I analyzed the DNA sequences of an intron-spanning region of the gene *EF1α* (Elongation factor 1 alpha) to identify shared nucleotide polymorphisms only present in diseased individuals. Some neoplastic individuals contained more than two alleles, and normal individuals contained only two. Because bivalves are diploid organisms, I hypothesize the excess alleles belong to a cancer cell non-native to the host. Neoplastic individuals showed the presence of a common allele, giving evidence of horizontal transmission of a clonal cancerous cell. Further DNA sequence analysis indicated that the *Mytilus chilensis* potentially has a transmissible cancer lineage independent from the cancer lineage found in *Mytilus trossulus*, the blue mussel native to the Northern Pacific. The results of this study suggest that the evolution of transmissible cancers in the ocean are more common than previously thought.

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Introduction

Cancer is a widespread disease in which cells accumulate mutations and divide uncontrollably. Cancer cells can destroy body tissue and disrupt homeostasis, shortening the lifespan of the organism. Cancer is not typically considered a contagious disease. Even when an infectious agent causes cancer, the cancer cells arise by the transformation of the somatic cells from the host and the cancer itself is limited to the diseased individual. A few examples include, the case of the Human Papillomavirus (HPV) which can cause multiple types of cancer (Gillison et al., 2000, Scudellari 2013) and the feline leukemia virus (FeLV), caused by a retrovirus that causes immunosuppression in cats, usually developing multiple types of cancer (Stuke et al., 2014). There is evidence of cases where cancer cells become contagious and are horizontally transferred between individuals in a population (Pearse and Swift 2006, Murgia et al., 2006, Metzger et al., 2015). As a consequence, cancer can act like a parasite and is no longer constrained by viral transmission or senescence.

Currently, there are only three known types of infectious cancers in nature. There are two in mammals, the Tasmanian devil facial tumor disease (DFTD) (Pearse and Swift 2006) and the canine transmissible venereal tumor (CTVT) (Murgia et al., 2006) and one in invertebrates, the bivalve transmissible neoplasia (BTN) (Metzger et al., 2015). In each type, the cancerous cells are derived from a single clonal origin which is genetically distinct from the infected host. The cancerous cells are transmitted horizontally because they can invade other hosts by evading their immune system. DFTD is particularly interesting because its

spread is threatening the species with extinction (Stammnitz et al., 2018). This cancer spreads when an infected devil encounters a healthy devil and bites it, implanting living cancer cells into a healthy individual. On the other hand, CTVT targets domestic dogs throughout the world and is transmitted by coitus. This cancerous cell line is, as far we know, the oldest and most widely spread cell line in the world, probably originating 11,000 years ago in an ancient breed of dog (Murchison et al., 2014).

Marine bivalves have two main types of cancer, gonadal neoplasia or germinoma, and disseminated neoplasia. The gonadal neoplasia is the proliferation of undifferentiated germ cells. This condition was first described in clams (Yevich, Barry, 1969) and later discovered in scallops (Peters et al., 1994) and mussels (Alonso et al., 2001). Disseminated neoplasia, on the other hand, is the proliferation of neoplastic cells that circulate in the organism. Molluscs have an open circulatory system where the heart pumps circulatory fluid known as hemolymph which bathes the internal organs. Hemocytes are the circulating cells in the hemolymph and are responsible for delivering nutrients and gases to the tissues, waste disposal, and defending against pathogens.

In disseminated neoplasia, malignant non-functional hemocytes proliferate and outcompete the population of normal hemocytes, impairing the physiological functions of the individual and potentially resulting in death of the organism (Cooper, Brown & Chang 1982). Disseminated neoplasia is diagnosed by the extraction of hemolymph and its histopathological examination. Neoplastic hemocytes show common morphological characteristics such as hypertrophy, the

presence of a hyperchromatic pleomorphic nucleus, swollen mitochondria, altered Golgi apparatus, and lack of phagocytic behavior and adhesion (Barber 2004). While normal cells in bivalves are diploid, cancer cells can show varying ploidy levels (Lowe & Moore 1978, Elston et al., 1990). Because neoplastic cells show higher DNA content, disseminated neoplasia can also be diagnosed by using flow cytometry to compare the DNA content of neoplastic and normal cells (Barber 2004).

The first report of disseminated neoplasia was in 1969 (Farley 1969). Multiple bivalves were diagnosed but most cases were associated with low prevalence of the disease (Mateo et al., 2015). However, an incident of higher prevalence and mass mortalities of the soft-shell clam, *Mya arenaria* on the Atlantic coast of North America motivated research about the nature and spread of this condition (McGladdery et al., 2001). Even though disseminated neoplasia has been studied since the 1970s as a conventional cancer, it was recently discovered that this disease is caused by a horizontally transmissible cancer that affects multiple marine bivalves (Barber 2004, Metzger et al., 2015). In contrast to the mammalian transmissible cancers, bivalves are sessile animals that do not require direct physical contact to become infected (Mateo et al., 2015). While it is currently not clear how the disease is spread between individuals, it is thought that transmission occurs through the engraftment of a cancerous cell while filter feeding (Metzger et al., 2015).

The discovery of transmissible cancers in four marine bivalves showed that transmissible cancers are present in the ocean, but little is known about the global

spread of this cancer (Metzger et al., 2016). Previously, it was shown in the marine mussel *Mytilus trossulus* off the coast of British Columbia that the genotype of the neoplastic hemocytes was discordant from the genotype of the host. When the genotype of neoplastic cells from different individuals was compared, it was found identical, indicating that cancer cells were from clonal origin and transmitted horizontally between individuals. The genotype of the neoplastic cells in *M. trossulus* was independent from neoplastic genotypes in other species, indicating that the original cancer cell was different from other cancer cells present in other species (Metzger et al., 2016). There have also been reports of disseminated neoplasia in other *Mytilus* species around the globe: *M. chilensis* along the Patagonian coast (Campalans, Gonzalez & Rojas 1998), *M. galloprovincialis* in the Slovene Adriatic Sea (Gombač et al., 2013) and *M. edulis* on the coast of Oregon (Farley 1969). However, it is unknown if the disseminated neoplasia present in these species spreads as a contagious cancer.

In this study, I sought to determine if the disseminated neoplasia present in *M. chilensis* is a transmissible cancer or a conventional cancer. Because *M. chilensis* does not have an available genome sequence, I used an available sequence of the gene *EF1α* (Elongation Factor 1 alpha), a ubiquitous protein involved in eukaryotic translation, which was previously used to screen for single nucleotide polymorphisms (SNPs) in the congeneric species *M. trossulus.* I amplified an intron-spanning region of this gene from both neoplastic and unaffected animals and observed more than two alleles in some diseased individuals. Neoplastic individuals showed the presence of two shared alleles that

were absent in all normal individuals. I then asked whether the transmissible cancers in *M. chilensis* and *M. trossulus* originated from the same cancerous cell, suggesting a single origin of the transmissible cancer, or whether the cancer present in *M. chilensis* originated independently, indicating that transmissible cancers have arisen multiple times in this group. The cancer genotypes present in *M. chilensis* and *M. trossulus* are dissimilar, indicating that these cancer lineages probably evolved as independent events. This study suggests that transmissible cancers are present in the southern hemisphere and may be more abundant than previously thought.

Materials and Methods

Sample collection and diagnosis

In February 2012, a team of marine biologists from Argentina collected a sample of 60 market-sized mussels (mean=67.6 mm; range=52–92 mm along the longest axis) was collected in February 2012 from a culture at Bahía Brown (54º52´S, 67º31´W), Beagle Channel, Argentina. The soft parts of the specimens were carefully removed from their shells and fixed in Davidson's solution (Shaw & Battle, 1957) for 24 hours. Oblique transverse sections, approximately 5 µm thick and including parts from each specimen's mantle, gills, gonads, digestive glands, nephridia, and foot. Tissue samples were embedded in paraffin, and 5 mm sections were stained with hematoxylin and eosin stain. Histological sections were examined using a Leica DM 2500 light microscope for the presence of neoplastic

cells. Small samples taken from the gills of each mussel were preserved in 100% ethanol for molecular analyses.

DNA extraction

DNA was extracted by Dr. Metzger from ethanol-fixed gill and siphon samples using DNeasy Blood & Tissue Kit (Qiagen) with the following additional steps to remove PCR-inhibiting polysaccharides. After tissue lysis, 65 µl of P3 Buffer (Qiagen) was added for 5 minutes to precipitate polysaccharides from the genomic DNA and centrifuged at 17k × g at 4°C for 5 minutes. The resulting supernatant was transferred to a new tube, 200 µl of Buffer AL was added, and the manufacturer's protocol resumed. These samples are a composite of hemolymph and solid tissue.

Molecular experiments

I targeted an intron spanning region of the gene *EF1α* (Elongation factor 1 alpha) which was previously used to screen for SNPs (single nucleotide polymorphisms) in other diseased species with BTN (Metzger et al., 2016). To identify SNPs within the *M. chilensis EF1α* gene, primers were designed on the exons of the gene and targeted an intron. Because the *M. chilensis* genome has not been sequenced, the available *EF1α* sequences for the congeneric species *M. trossulus* and *M. edulis* were used as reference to design the primers. The targeted region was amplified using polymerase chain reaction (PCR) with PfuUltra II Fusion HS DNA Polymerase (Agilent). The PCR products were gel-extracted using spin columns (Qiagen, NEB) and directly sequenced. When multiple alleles were

found at a locus, the PCR products were cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). Plasmids containing inserts were transformed into TOP10 or DH5α competent *E. coli* (Invitrogen), and 5-12 clones were sequenced using M13F and M13R primers (Genewiz). Multiple clones were screened for each individual to screen for the different SNPs, insertion and deletions present. The DNA sequences for *M. chilensis* were then compared to the cancer genotypes seen in the congeneric species *M. trossulus* and *M. edulis.*

To identify if there was evidence of horizontal transmission, primers were designed to amplify alleles that were present in neoplastic individuals but absent in normal individuals. qPCR was carried out using PowerUp SYBR Green Master Mix (Applied Biosystems) on neoplastic and normal samples. For the qPCR, one primer set amplified only a cancer-associated allele, whereas a second pair of primers amplified all *Mytilus* alleles in *EF1α*.

Results

To determine if the disseminated neoplasia in *M. chilensis* is a conventional cancer or a transmissible cancer, I amplified an intron-spanning region of the gene *EF1α* and used these sequences to assess the genetic relatedness between different individuals. The sequencing data from two of the three neoplastic individuals showed more than two alleles, suggesting the presence of neoplastic cells independent from the host cells. Since normal cells are diploid, the presence of additional alleles potentially belong to the cancer cells, indicating the presence of neoplastic cells independent from the host (**Figure 1**).

I then identified two allele sequences that were only present in two diseased individuals. To test if the alleles were present in all neoplastic individuals, we designed primers targeting the nucleotide polymorphisms present in those sequences (**Figure 2**). The PCR showed amplification in all neoplastic samples. (**Figure 3**). Because one of the neoplastic samples showed low amplification of the cancer-associated allele, we hypothesized all neoplastic samples have the, but in different amounts. Therefore, I decided to use qPCR to confirm that all the neoplastic samples have the cancer-associated allele.

The qPCR results (**Figure 4**) showed a much higher copy number in neoplastic samples compared to normal samples, confirming that the cancer-associated allele is present in all neoplastic samples and undetectable in normal samples.

I hypothesized that the differences in cancer-specific DNA content in individuals with neoplastic cells could reflect that the samples have different numbers of neoplastic cells circulating in the hemolymph, perhaps because some animals have a more advanced cancer. Unfortunately, because the samples are composed of hemolymph and solid tissue, and not separate samples of hemocytes and solid tissue, I am not able to compare directly the amount of DNA of neoplastic cells to the amount of DNA of normal cells between neoplastic animals to test this hypothesis. This direct comparison would give us inference about how advanced the neoplasia was, well as which alleles belong to the host and which to the neoplastic cells.

Discussion

Here, I present evidence of a transmissible cancer in *M. chilensis*. Based on the DNA analysis of the gene *EF1α*, I hypothesize that the cancer in this species is spreading through horizontal transfer of clonal cancerous cells. If the cancer would have been conventional, it would be very improbable to see the same alleles among different neoplastic individuals and absent in normal individuals. This finding strongly argues that transmissible cancers in the ocean are not only present in the northern hemisphere but also in the southern hemisphere, making contagious cancers in the ocean more widespread than previously thought.

Further studies of the sequences revealed that the cancer alleles of *M. chilensis* and *M. trossulus* are dissimilar, suggesting that the cancer lineage observed in *M. trossulus* is a different cancer lineage than the one seen in *M. chilensis* (**Figure 5**). Moreover, it is particularly interesting that the cancer lineage that we found on the coast of Patagonia is identical to the one seen in *Mytilus edulis* on the Atlantic coast of France. There is evidence of convergent evolution in multiple types of cancer that became resistant to certain treatments because the cancer cells acquired the same mutations (Hanahan and Weinberg 2011, Fortunato et al., 2017). Nevertheless, a more plausible explanation is the evolution of a transmissible cancer, which subsequently diverged into multiple sub-groups. We hypothesize that the transmissible cancer present in *M. chilensis* and *M. edulis* came from the same cancer cell and spread through horizontal transmission. This disease was potentially dispersed by human trade since Chile is one of the largest producers of mussels in the world and Europe has a particularly high consumption

and trade rate. Since it is possible for mussels to be transported in the ballast water of ships and invade other suitable environments (Naddafi et al., 2011), I hypothesize this transmissible cancer lineage arose either in Chile or France and was then carried to the other location.

Even though little is known about how transmissible cancers arise, the transmission of cancer cells probably resembles an analogous mechanism to the metastatic process seen in conventional cancers. Hence, the transmission of cancer cells from one individual to another is a metastatic event (Siddle 2017). In order to metastasize, cancer cells have to undergo complex adaptations often leading to an aggressive phenotype (Murchison 2009). Natural selection favors adaptations like detachment from the primary tumor and tissue invasion since cancer cells are able to grow and divide more quickly (Fidler 2003, Fortunato et al., 2017). However, the acquisition of these adaptations is an evolutionary dead end because cancer cells harm the host, shortening the lifespan of the individual and therefore causing the extinction of the cancer (Fortunato et al., 2017).

Transmissible cancers, however, have solved this problem because the cancer cells are no longer constrained to one host. This allows the cancer cells to continue evolving until the host becomes resistant or extinct. One example is the transmissible cancer present in golden carpet shell clams, *Polititapes aureus,* which originally arose from pullet shell clams, *Venerupis corrugata.* This was found based on DNA sequence analysis, where the genotype of the cancerous cell in *P. aureus* resembled the genotype of *V. corrugata* rather than its own. This cancer has not been detected in *V. corrugata,* probably because the population that was

susceptible to the cancer went extinct, leaving only the population resistant to the cancer. (Metzger et al., 2016). There is also evidence that some Tasmanian devils have antibodies against DFTD1 (one of the cancer lineages of DFTD) and are potentially developing resistance against this disease. (Murchison et al., 2010). This host-cancer interplay can become an evolutionary arms race where the host will evolve to become resistant and the cancer cell evolve to overcome resistance, potentially leading to a dynamic coevolution where neither goes extinct. On the other hand, if transmissible cancers evolve to cross the species barrier, transmissible cancers could evolve a more aggressive phenotype since the extinction of the host species does not entail the extinction of the transmissible cancer.

Interestingly, transmissible cancers are capable evading and suppressing the immune system. In mammals, tumor growth is attained by the down-regulation of the antigen processing pathway, suppressing the expression of MHC (major histocompatibility complex) proteins (Siddle et al., 2013). This allows the cell to not be detected and start growing without triggering an immune response.

Because Tasmanian devils are an endogenous species of the island of Tasmania, the population has low genetic diversity. It is hypothesized that because of this low diversity, the immune system is unable to recognize the allograft, allowing the spread of the cancer to other individuals (Jones et al., 2004, Siddle 2007). However, a recent study showed evidence of strong positive selection of genes associated to immune function or cancer risk in mammals, causing rapid evolution towards resistance to DFTD (Epstain et al., 2016). In the case of dogs,

selective breeding has caused genetic bottlenecks resulting in low genetic diversity, allowing transmission of the cancer cell without immune rejection (Jones et al., 2004, Siddle et al., 2013). CTVT progress, however, depends on the balance between tumor growth and tumor suppression mediated by the immune system, usually leading to a stationary phase of the disease (Siddle et al., 2013).

While the MHC is thought to have an important role in cancer transmission in vertebrates, there is no evidence of the presence of a MHC in invertebrates (Loker et al., 2004). Moreover, while histocompatibility is known to exist in some species of marine invertebrates, nothing is known about whether these systems operate in bivalves. (De Tomaso et al., 2005, Dishaw & Litman 2009). The results of this study give evidence of the discovery of an independent lineage of transmissible cancer in bivalves. Nevertheless, it remains to be discovered if the engraftment of the cancer cell triggers an immune reaction in the host. Further studies could determine if the presence of an immune reaction is the basis of the interspecific barrier of transmission.

Conclusion

This study suggests that the disseminated neoplasia in the Chilean blue mussel, *Mytilus chilensis,* is a transmissible cancer. Here, we found evidence of the presence of more than two alleles in neoplastic samples with two shared alleles only present in diseased individuals. This indicates that the neoplastic cells are most likely from clonal origin and spread through horizontal transfer among

individuals. The discovery of a new transmissible cancer widens the possibility of the presence of more transmissible cancers in nature and possibly in humans.

Figures and Tables

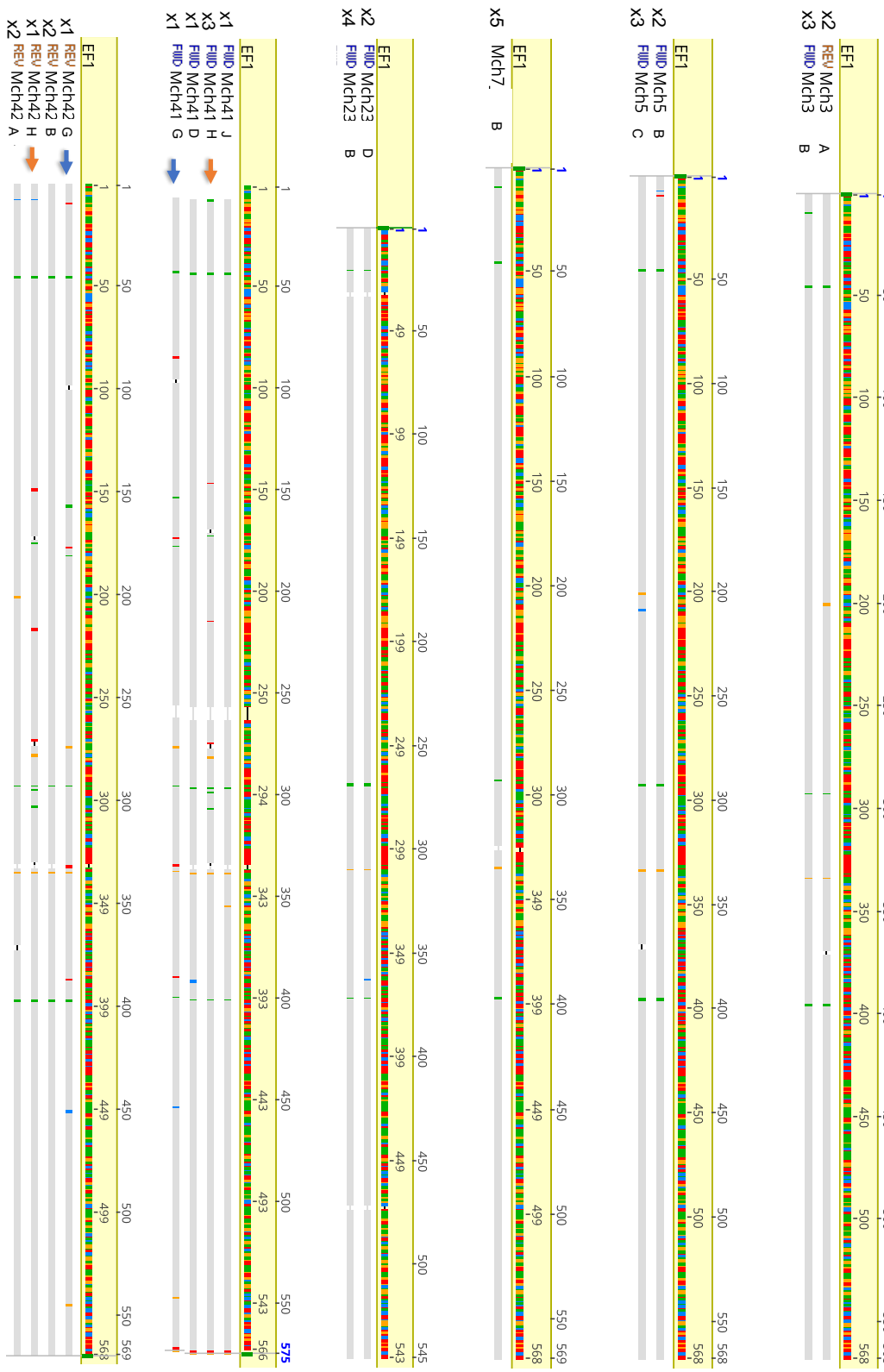

Figure 1. Different allele sequences found in the intron-spanning region of the gene *EF1α*. Point mutations are shown as color changes. These sequences were obtained using Sanger sequencing of plasmids. Six individuals were studied Mch3, Mch5, Mch7 are samples from normal individuals and Mch23, Mch41, Mch42 are samples from neoplastic individuals. Two of the three neoplastic individuals show the presence of more than two alleles. Since the clones were sent for sequencing repeatedly, the number on the left of each sequence represents the number of times the sequence was obtained. These sequences were later analyzed to detect the presence of an allele only present in diseased individuals. Samples from individuals Mch41 and Mch42 share two alleles G and H, denoted by blue and orange arrows respectively. The presence of alleles G or H was not detected in Mch23 by clone sequencing.

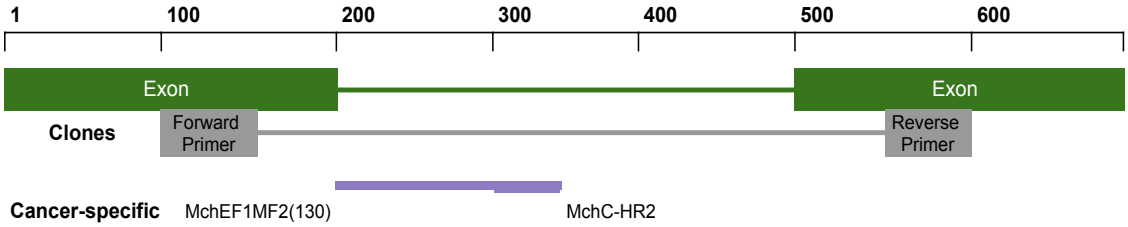

Figure 2. Visual representation of the DNA sequences in the intron spanning region of the gene *EF1α*. The scale bar is in base pairs. The primers targeting the intron spanning region were designed on the exons. Allele H is shown in purple with its corresponding primers on either side. Allele H was only found in diseased individuals.

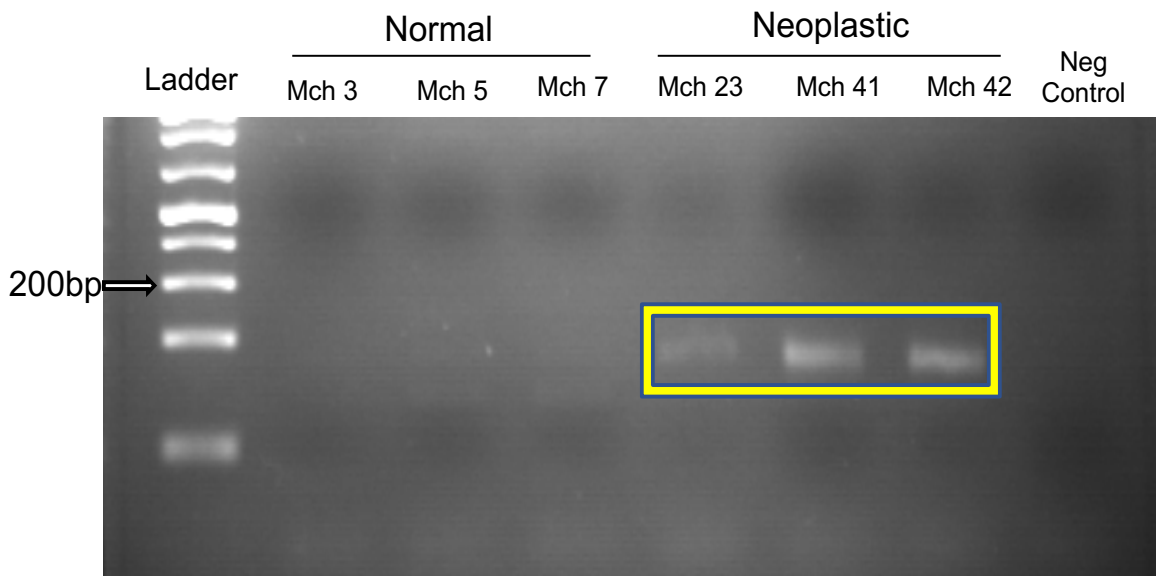

Figure 3. To determine the presence of horizontal transmission of cancer cells, we amplified a section of about 170bp in the intron-spanning region of the gene *EF1α* using polymerase chain reaction (PCR) and agarose gel electrophoresis. This reaction amplified an allele present in all neoplastic individuals (Mch23, Mch41, Mch42) and absent in all normal individuals, suggesting the presence of horizontal transmission of clonal cancer cells between individuals. These results were later confirmed with quantitative polymerase chain reaction (qPCR).

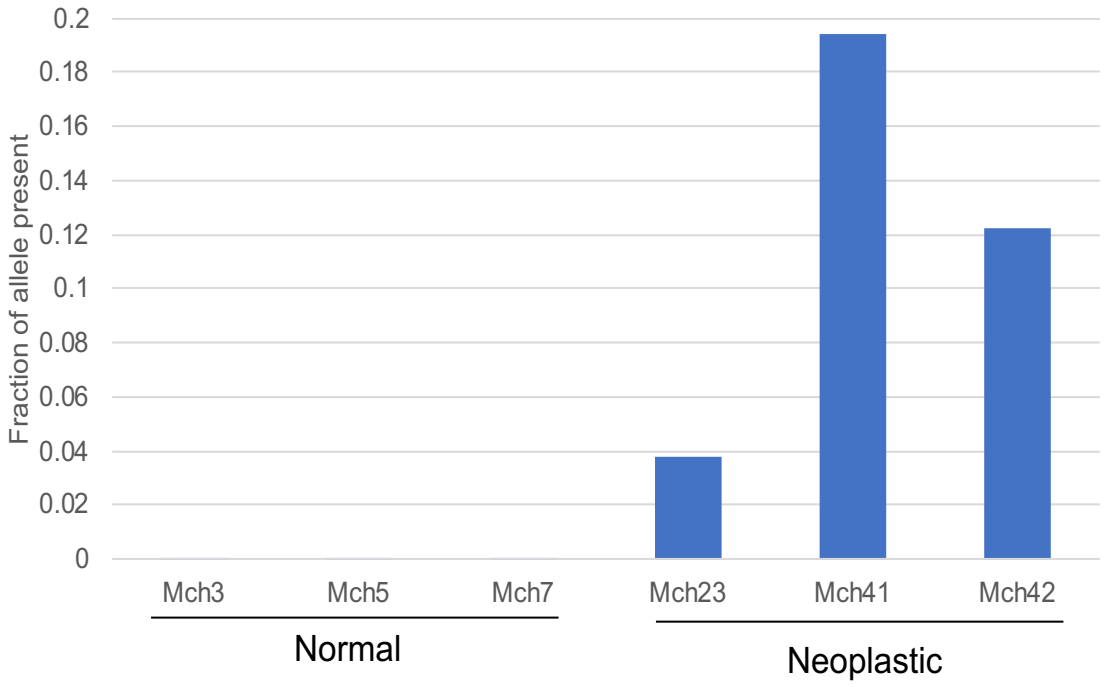

Figure 4. The quantitative polymerase chain reaction (qPCR) results for the cancer-associated allele of gene *EF1α* show a much higher copy number of the cancer-associated allele in neoplastic individuals (Mch23, Mch41, Mch42) than in normal individuals. This reaction was performed to confirm the results obtained with polymerase chain reaction (PCR) and agarose gel electrophoresis. Sample Mch41 has the highest copy number, followed by Mch42 and lastly, Mch23.

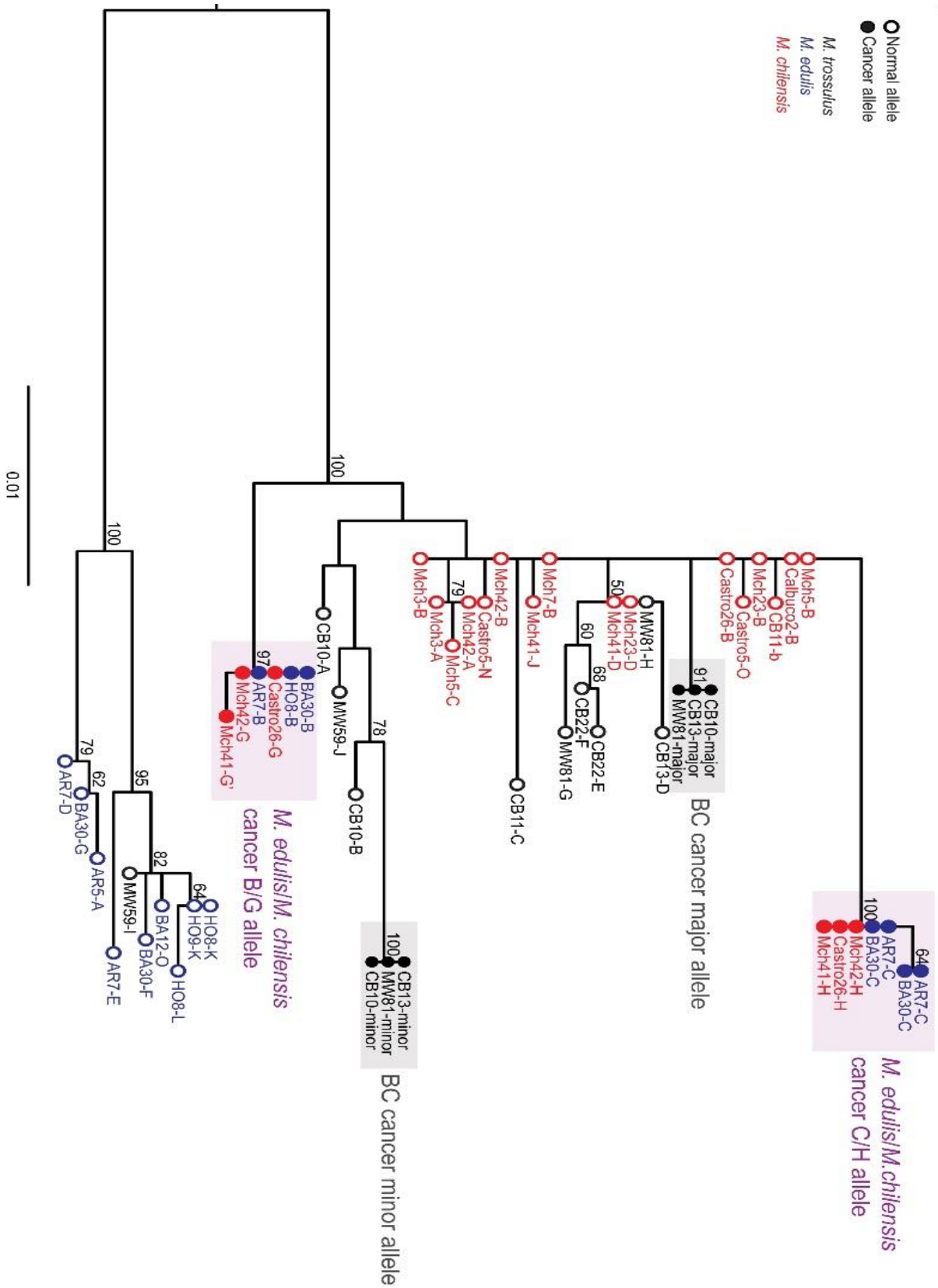
Normal allele
Cancer allele
M. trossulus
M. edulis
M. chilensis
Mch5-B
Calbuco2-B
CB11-b
Mch23-B
Castro5-O
Castro26-B
MW81-H
Mch23-D
Mch41-D
CB22-F
MW81-G
CB22-E
CB11-C
Mch7-B
Mch41-J
Mch42-B
Castro5-N
Mch42-A
Mch5-C
Mch3-B
Mch3-A
CB10-A
BA30-B
HO8-B
Castro26-G
AR7-B
Mch42-G
Mch41-G'
MW59-J
CB10-B
CB10-major
CB13-major
MW81-major
CB13-D
BC cancer major allele
CB13-minor
MW81-minor
CB10-minor
BC cancer minor allele
AR7-C
BA30-C
Mch42-H
Castro26-H
Mch41-H
M. edulis/M.chilensis
cancer C/H allele
M. edulis/M. chilensis
cancer B/G allele
HO8-K
HO9-K
HO8-L
BA12-O
BA30-F
MW59-I
AR7-E
AR5-A
BA30-G
AR7-D
0.01

Figure 5. Normal and neoplastic animals from the following locations were analyzed: *Mytilus trossulus* from British Columbia is shown in black, *Mytilus chilensis* from Argentina is shown in red, and *Mytilus edulis* from France is shown in blue. Names specify individual ID and allele ID. Open circles denotes alleles from normal individuals. Closed circles mark cancer-associated alleles. The tree was rooted at the midpoint, with bootstrap values below 50 removed. Model used was HKY85+G. The scale bar marks genetic distance.

Forward Primer	
Mch-EF1MF1(156)	TTACTCCTTGAGGGTTTTAGTATACT
Mch-EF1MF2(155)	ATGACTCCTTGAGGGTTTAT
Mch-EF1MF2(130)	TGGCTGAAAACCAGATTCTA
Reverse Primer	
Mch-EF1MR1 (579)	CTCTGGTCTGACCGTTGGAC
Mch-EF1MR2(298)	ATTTCTTTTAGTCACACAAT
MchC - HR2	AAAATTTCTTTTAGTCACACAAT

Table 1. The cancer-associated allele in the intron-spanning region of the gene *EF1α* was targeted by the forward primer Mch-EF1MF2(130) and reverse primer MchC - HR2. The primer pairs Mch-EF1MF1(156), Mch-EF1MR1 (579) and Mch-EF1MF2(155), Mch-EF1MR2(298) showed nonspecific binding, targeting both normal and neoplastic individuals.

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