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Review

Amplification of Butyrylcholinesterase and Acetylcholinesterase Genes in Normal and Tumor Tissues: Putative Relationship to Organophosphorous Poisoning

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Cholinesterases are ubiquitous carboxylesterase type B enzymes capable of hydrolyzing the neurotransmitter acetylcholine which are transiently expressed in multiple germline, embryonic, and tumor cells. The acute poisoning effects of various organophosphorous compounds are generally attributed to their irreversible covalent interaction with cholinesterases and block of their catalytic activities. We have recently found a de novo inheritable amplification of a CHE gene encoding defective butyrylcholinesterase (acylcholine acyl hydrolase; EC 3.1.1.8) in a family under prolonged exposure to the agricultural organophosphorous insecticide methyl parathion. Further analysis revealed that both the CHE and the ACHE genes, encoding acetylcholinesterase (acetylcholine acetyl hydrolase; EC 3.1.1.7), are amplified in leukemias and platelet disorders and that the tumorogenic expression of these genes in ovarian carcinomas is associated with their frequent coamplification in these tumors. The amplification of CHE and ACHE genes in normal and tumor tissues might be analogous to the well-known amplification of other genes encoding target proteins to toxic compounds. As such, it could provide cells a selection advantage when exposed to organophosphorous poisons. Further, since cholinesterases appear to play developmentally important roles in multiple cell types, the amplification and overexpression of their corresponding genes might affect fertility, be related to the progression of various tumor types, and bear upon the ecological and clinical risks involved with the common use of organophosphorous poisons.

KEY WORDS: DNA amplification *in vivo;* spermatogenesis; organophosphorous poisons; cholinesterases; tumorogenesis.

INTRODUCTION

Cholinesterases Play Pivotal Roles in Breathing and Are the Target for Organophosphorous Poisons

Cholinesterases are ubiquitous, polymorphic carboxylesterase type B enzymes capable of hydrolyzing the neurotransmitter acetylcholine and numerous ester containing drugs . In neuromuscular junctions and neuronal cholinergic synapses they take part in terminating neurotransmission (1). Covalent binding of organophosphorous compounds to the serine residue in the active esteratic site of these enzymes results in their complete, irreversible inactivation (2). Consequently, blockage of cholinesterases by organophosphorous nerve gases or commonly used agricultural insecticides induces serious acute and delayed poisoning effects. These include damage to the peripheral and central nervous system, myopathy, psychosis, general paralysis, and death (3). Estimations are that 19,000 deaths occur of the 500,000 to 1 million annual reported pesticide-associated poisonings, and unreported subacute intoxication is likely to mount to much higher numbers (4).

The two major types of cholinesterases are acetylcholinesterase (acetylcholine acetyl hydrolase; ACHE; EC 3.1.1.7) and the less substrate-specific butyrylcholinesterase (acylcholine acyl hydrolase; CHE; EC 3.1.1.8). In humans, cDNA probes were isolated for both (Refs. 5 and 6, respectively). Genetic linkage analysis has mapped the functional CHE gene to the long arm of chromosome 3, and CHEcDNA was shown to hybridize with genomic DNA sequences on chromosomes 3 and 16 (7,8).

Mutations in the CHE gene appear to be linked with postanesthetic apnea, caused by the inability of defective CHE to destroy the muscle-relaxant drug succinylcholine (9). Such mutations also result in a particularly high sensitivity to organophosphorous poisons, caused by inefficient scavenging of these compounds in individuals with defective CHE (10).

Developmentally Related Expression of Cholinesterase Genes

The genes encoding CHE and ACHE are transiently expressed in multiple embryonic cell types in a manner sug-

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gesting developmentally related function(s) for these enzymes. In humans, examples include *oogenesis,* where both the enzymatically active proteins and their mRNA transcripts were found to be abundant (11,12); *spermatogenesis,* where cholinesterases were implicated with sperm motility (13); *embryogenesis,* where secretion of cholinesterases into the amniotic fluid is clinically accepted as a marker of neural tube closure defects (1); *hemocytopoiesis,* where both erythrocytes (14) and promegakaryocytes (15) are noted by production of ACHE, and *tumorogenesis,* where cytochemical staining (16) or biochemical measurements (17) have demonstrated in various tumor types overproduction of cholinesterases as compared with the corresponding normal tissues, as well as abnormal properties of serum CHE in carcinoma patients (18). Table I summarizes the multiple developing biosystems where cholinesterase genes are expressed, demonstrating their diverse production in differentiating human cells of various lineages.

Cholinesterase Gene Amplification May Provide Selection Advantage for Cells or Tissues Exposed to Organophosphorous Inhibitors

The recurrent expression of cholinesterase genes in embryonic or undifferentiated cells can potentially reflect a functionally important role(s) for these enzymes. Therefore, it is conceivable to assume that inhibition of cholinesterase activities could be harmful to these systems. As stated above, this effect would be particularly severe in individuals with defective CHE genes. In several studies on other metabolically important proteins, the amplification of the genes encoding these proteins provided cells or tissues with selection advantage under exposure to their respective toxic inhibitors. Well-known examples are the amplifications of the DHFR gene, encoding dihydrofolate reductase, in methotrexate-treated leukemias (21) and the thymidilate synthetase gene in tumors treated with fluorodeoxyuridilate (22). Table II lists these and several other examples of amplification of metabolically important genes under cytotoxic inhibition. By drawing the analogy between these genes and those encoding cholinesterases, one would expect the cholinesterase genes to amplify under exposure to organophosphorous poisons. In the following, recent findings supporting this hypothesis are summarized and discussed.

Table I. Developmentally Regulated Expression of Cholinesterase Genes in Various Human Cell Types

Cell type	Supporting evidence	Ref. No.
1. Immature oocytes	*In situ* hybridization	11
	Biochemical measurements	12
	Indirect biochemical analyses	13
2. Developing sperm	*In situ* hybridization, RNA blot hybridization	Unpublished data
3. Neural tube	Amniotic fluid, Biochemical measurements	19
4. Hemopoietic stem cells	Cytochemical staining, Biochemical evidence	15
5. Undifferentiated ovarian carcinomas	Cytochemical staining	16
	RNA blot hybridization, *In situ* hybridization	20

EXPERIMENTAL OBSERVATIONS

Inheritable Amplification of Defective Cholinesterase Genes in Individuals Exposed to Organophosphorous Insecticides

The first case of CHE gene amplification was found in a family of farmers with the defective "silent" CHE phenotype who were under repetitive exposure to the agricultural organophosphorous insecticide parathion (*P*-nitrophenyldiethylthionophosphate). The H. family parents were employed in agricultural work and exposed to parathion since 1950. Several mild incidents of organophosphorous intoxication to the mother were symptomatically treated. Routine CHE tests, run in later years by the health authorities, indicated particularly low CHE activities in the serum of one of their sons.

When, in spite of instructions, this son took part in Parathion spraying, he displayed acute characteristic apnea (23) and was hospitalized and artificially respirated. A daughter of this family suffered from infertility problems and postanesthetic apnea following succinylcholine administration during the course of a laparoscopy. Serum CHE measurements demonstrated low butyrylthiocholine hydrolytic activities, high sensitivity to organophosphorous CHE inhibitors, and resistance to dibucaine for the serum butyrylcholine hydrolytic activities from both daughter and son, as expected from "silent" CHE (10). Relatively low activities were also found in the serum of one of the son's children. Upon DNA blot hybridization, peripheral blood DNA from this son and his child was found to contain about 100 copies (quantified by dot blots of six dilutions from each DNA) of a genomic DNA fragment hybridizing with CHEcDNA. All of the other members of the family, including the parents and the daughter, displayed completely normal hybridization patterns and intensities.

Detailed karyotype analysis of lymphocyte chromosomes from these family members revealed apparently normal G and Q banding patterns. There were no abnormal extrachromosomal structures, characteristic of unstable amplified genetic material, or homogeneously stained regions commonly observed in chromosomes containing large amplification units (24). *In situ* hybridization of lymphocyte chromosomes from these individuals with ^{35}S-labeled CHEcDNA revealed intensive labeling in the 3q26-ter region in chromosomes from the individual having the amplified CHE gene, whereas the labeling on chromosomes from other family members was indistinguishable from controls (23). Thus, the amplified CHE gene appears to have been stably integrated at, or close to, its original position on chromosome 3 (7).

The absence of CHE gene amplification in the parents and its presence in one of their sons and a grandson, demonstrated the *de novo* appearance of an inheritable gene amplification or the predisposition for such an event (23). There is no recorded precedence for such phenomenon in humans, although gene amplification was found, as detailed above, in many genes of cultured cells and primary tumors (21,22).

Coamplification of Cholinesterase Genes in Leukemias and Platelet Disorders

The finding of a *de novo* inheritable amplification of the

Table II. Examples for Gene Amplifications Under Cytotoxic Inhibition

Inhibited protein	Blocked metabolic process	Cytotoxic inhibitor
1. Adenosine deaminase	Nucleic acid synthesis	Deoxycoformycine
2. ATPase	Energy production	Ouabain
3. Dihydrofolate reductase	Thymidine synthesis	Methotrexate
4. Hydroxymethyl glutaryl (*O-A* reductase)	Sterol metabolism	Compoctine
5. Ornithine decarboxylase	Polyamine biosynthesis	Difluoromethylornithine
6. Ribonucleotide reductase	Nucleic acid metabolism	Hydroxyurea
7. Thymidilate synthetase	DNA synthesis	Fluorordeoxyuridilate
8. UMP synthetase	RNA synthesis	Pyrazuforin

CHE gene in individuals exposed to commonly used CHE inhibitors raised several questions. First, was this an incidental unrepresentative exception, or did it reflect a significant phenomenon and occur elsewhere too? Second, if CHE genes do amplify nonrandomly, would ACHE genes amplify as well, as expected from their functional relatedness? Third, could such amplification events take place in somatic or tumor cells in a noninheritable manner, and if so, would this be related with specific clinical implications?

To answer these questions, we searched for evidence on diseases in which nonrandom aberrations occur in the 3q26-ter site where we mapped the CHE gene, since gene amplifications and chromosome breakage appear to be associated phenomena in multiple tumor types. We found that the 3q26-ter region is frequently subjected to deletions, inversions, and translocations in acute myelodysplastic leukemias (25). Moreover, patients having such breakages all featured enhanced magakaryocytopoiesis, altered platelet counts and rapid progress of the disease (26). That was particularly intriguing, since the administration of acetylcholine analogues and cholinesterase inhibitors has been shown to induce promegakaryocytopoiesis and enhance platelet formation in the mouse, both *in vivo* (27) and in bone marow cultures (28).

Based on this indicative correlation, we tested the cDNA probes for the CHE and the ACHE genes in blot hybridization with peripheral blood DNA from various leukemic patients. Ten- to two hundred-fold intensified hybridization signals and modified restriction patterns were observed with both cDNA probes in 4 of the 16 leukemia DNA preparations examined (29). These reflected the amplification of the corresponding ACHE and CHE genes and alterations in their structure. Parallel analysis of 30 control samples revealed nonpolymorphic, much weaker hybridization signals for each of the probes. In view of the above-discussed reports on the effect of acetylcholine analogues and CHE inhibitors in the induction of megakaryocytopoiesis and production of platelets in the mouse, we further searched for such phenomena in nonleukemic patients with platelet production disorders. Amplifications of both ACHE and CHE genes were found in three of the five patients so far examined. Pronounced coamplification of these two related but distinct genes in correlation with pathological production of blood cells therefore suggested a functional role for members of this gene family in megakaryocytopoiesis and raised the question whether the coamplification of these genes could be causally involved in the etiology of hemocytopoietic disorders.

Tumorigenic Expression of Cholinesterases in Ovarian Carcinomas Is Associated with Gene Amplification

The coordinated amplification of the CHE and ACHE genes in hemopoietic tumors indicated that this phenomenon is statistically significant, related to the function of cholinesterases in nondifferentiated cells and has biological and clinical implications. This, in turn, suggested that the same amplification phenomenon might occur in other tumor types as well. The best candidate tumors were those where enhanced expression of catalytically active cholinesterases has previously been noted, for example, ovarian carcinomas. We have previously found that in normal ovaries, the expression of cholinesterase genes was confined to oocytes alone (11,12), while cytochemical staining of ovarian tumors indicated extensive expression of these enzymes (16). Indeed, DNA blot hybridization demonstrated a four- or more-fold amplification of both the CHE and the ACHE genes in 6 of 11 malignant carcinomas of the ovary that were studied (20), whereas no such amplification was observed in normal ovarian tissues, benign ovarian cysts, or control tissues from the patients having amplified cholinesterase genes in their tumors. In fact, all of the ovarian tumors which had amplified oncogenes such as C-RAFI, V-SIS, and C-FES also displayed high intensities of hybridization with the cholinesterase probes; moreover, three malignant tumors were found to have coamplified cholinesterase genes but no amplified oncogenes, perhaps indicating that the event of cholinesterase gene amplification precedes that of the oncogenes in tumor progression.

Xenopus oocyte microinjections of ovarian tumor mRNA demonstrated the presence of translatable cholinesterase mRNAs, as was confirmed by blot and *in situ* hybridizations and by immuno- and cytochemical staining of tumor frozen sections. Interestingly, there appeared to be tumor foci with small, rapidly dividing cells that were particularly active in expressing the amplified genes (20). The frequent coamplification of the CHE and ACHE genes in ovarian *and* hemopoietic tumors therefore suggested that cholinesterases might be involved with neoplastic growth and/or proliferation as is summarized in Table III.

CONCEPTUAL CONSIDERATIONS

Analogy of Cholinesterase Gene Amplifications to Those of Other Genes Encoding Target Proteins to Toxic Compounds

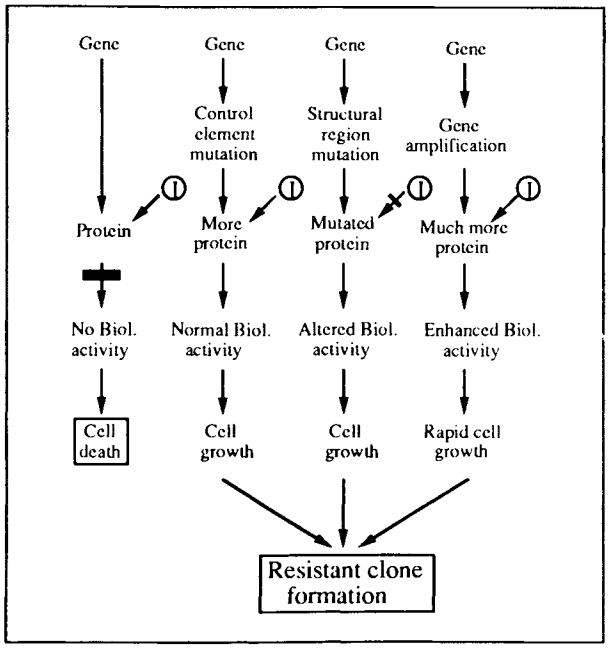
Following the discovery of DHFR gene amplification

under methotrexate chemotherapy, it was further revealed that various genes amplify under exposure of cells in culture or of primary tumors in treated patients to specific inhibitors. This provides survival advantage to cells with amplified genes for the target proteins of these inhibitors. Our experimental observations imply that the cholinesterase genes fall into this category, assuming that their protein products are indeed important for the survival, division, or growth of particular tumor, hemopoietic, and germline cells.

Gene amplification is not the only mechanism enabling survival of cells under exposure to toxic compounds. Other options are (a) mutations that would prevent the inhibitor from binding its target by occurring in the structural domains of the genes encoding the target proteins (31), (b) mutations in the promoter region or in *cis*- or *trans*-regulatory proteins that would enhance the production of this target protein without the need for direct gene amplification (32), and (c) altered transport through the cells' membrane that would prevent the inhibitor from getting to its target protein, as in multidrug resistant cells (33). Scheme I summarizes these protective mechanisms.

Assuming that the analogy stated above is justified, one expects that the other mechanisms for bypassing organophosphorous inhibition should operate as well. Interestingly, we have recently found structurally altered CHE genes in neuroblastoma and glioblastoma tumors (34) that encode a CHE protein with considerably weaker binding constants to organophosphorous inhibitors, as compared to those of the normal enzyme (Neville *et al.*, in preparation). Further studies will be required to determine whether the transcriptional activity of the CHE gene in particular tumor cells is upregulated by specific alterations in its promoter; however, the multidrug resistance mechanism may not apply to the extracellular CHE protein (35).

Alternative explanations for the phenomenon of cholinesterase gene amplification cannot yet be excluded for lack of information. For example, amplified and overexpressed genes coding for a number of oncogenes provide survival advantages to cells; however, there is no apparent homology between cloned DNA sequences in the genes coding for cholinesterases and known oncogenes. Other genes amplify simply because they are localized near a gene that gets amplified, since the size of core amplification units can be large enough to encompass more than one gene. To the best of our knowledge, there is no known oncogene in the vicinity of the CHE gene on the long arm of chromosome 3. Hence, the analogy to other genes encoding target proteins to inhibitors remains as the only current explanation for cholinesterase gene amplification that is supported by experimental evidence.



Scheme I

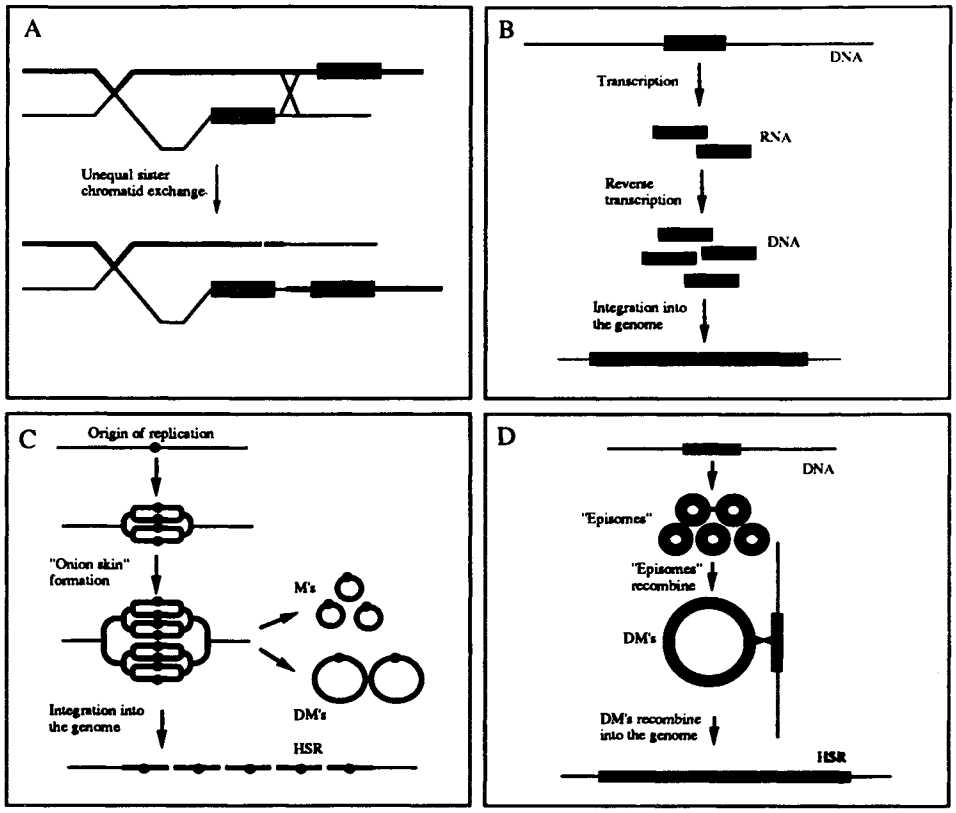
Possible Mechanisms for the Initial Amplification Event

Several mechanisms were proposed that could account for the initial event in the process of gene amplification. These include nonequal crossing-over in exchange of genomic material during cell division (22), reverse insertion of host sequences integrated into viral genomes (36), "onionskin" amplification around a single origin of replication (21), and the production of episomes, double minute chromosomes, and chromosome-inserted homogeneously stained regions (HSR) (37). Scheme II presents these putative mechanisms. It is not yet clear whether any of these, or any of their combinations, may be applicable for the initial

Table III. Amplification of Cholinesterase Genes in Germline and Tumor Cells[a]

Putative cell type where initial event occurred	Incidence	Cholinesterase presumed function	Ref. No.
1. Developing sperm	1 (inheritable) No known precedences	Providing survival advantage under parathion exposure	23
2. Myeloid progenitor cells	4 (of 16 examined)	Growth and/or differentiation related	29
3. Promegakaryocytes	3 (of 5 examined)	Induction of stem cell differentiation into megakaryocytes	30
4. Serous papillary adenocarcinoma of the ovary	3 (of 4)	Growth and/or proliferation	20
5. Other malignant ovarian carcinomas	3 (of 7)	Growth and/or proliferation	20

[a] It should be emphasized that in each of these examples, large numbers of control samples were examined in which no amplification was observed (see text for details).



Scheme II

amplification of cholinesterase genes in germ line and tumor cells.

The amplified CHE gene in its inheritable and tumor forms is apparently mutagenized, based on its restriction pattern (20,23). This is supporting evidence for the involvement of mutation in the amplification process. In addition, the variable *Eco*RI restriction pattern of the amplified genes (23) is reminiscent of an onionskin initial event (21) and its *in loco* insertion onto the Chr. The 3q26-ter position is agreeable with the *in loco* appearance of various oncogenes in HSR chromosomal domains (24). Finally, the expression of cholinesterase genes in developing sperm cells agrees with unequal crossing-over during meiosis—a process which may lead to the occurrence of gene amplification and the formation of HSR in normal circumstances, let alone exposure to toxic inhibitors. To examine this issue further, the human CHEcDNA sequence has been inserted into transgenic mice that are currently being exposed to controlled cholinesterase inhibition by organophosphorous poisons, hoping to delineate the origin of the amplification process and its defined mechanisms.

Ecological and Clinical Implications

Recent literature suggests that the exposure to commonly used agricultural organophosphorous poisons may be harmful to fetal development. Animal studies have demonstrated that methyl parathion administration suppressed growth and induced ossification in both mice and rats, as well as high mortality and cleft palate in the mouse. In humans, malformations of the extremities and fetal death were correlated with exposure to methyl parathion in 18 cases. In addition, a neonatal lethal syndrome of multiple malformations was reported in women exposed to unspecified insecticides during early pregnancy (38). Further studies should be performed to examine whether such damages are correlated with the occurrence of the "silent" cholinesterase phenotype and with the appearance of ACHE-CHE gene coamplifications.

Another, relatively neglected aspect of organophosphorous intoxications is the damage induced to chromosomes under such exposure. Recurrent and highly significant chromosome breakage appears in peripheral blood cells from 25% of the agricultural workers exposed o organophosphorous insecticides but was considered relatively unimportant in view of its transient (approximately 6-month) nature (39). Following our finding of amplified cholinesterase genes in peripheral blood cells from leukemic patients, one wonders whether the earlier reports on chromosome breakage did not, at least in part, reflect the appearance of amplified ACHE and CHE genes in these individuals as well.

The induction of chromosome breakage under organophosphorous intoxication might also occur in germline cells, for example, developing sperm, and be transferrd to the next generation. Such an event could potentially explain the inheritable amplification of cholinesterase genes in our first family (23). Another type of germline cells in which cholinesterase genes are expressed is the oocytes (11,12), which should also be vulnerable for changes in these genes. Furthermore, it has been shown that acetylcholine induces

meiotic maturation in *Xenopus* oocytes (for detailed discussion of this issue see Refs. 11 and 12), indicating that the cholinergic system is an important part of this biological process. A considerable increase in the concentration of acetylcholine, a direct consequence of environmental organophosphorous exposure, could hence serve as a signal for nuclear changes in the exposed germline cells and induce changes in their transcriptional activities. Several lines of evidence correlate gene amplification with gene expression, on one hand, and cell division, on the other. Agents or treatments inducing DNA damage increase the general incidence of gene amplification in the treated cells (21,22), and all of the models proposed to explain the mechanism of gene amplification relate it to DNA duplication processes (Scheme II). In view of the reported effects of acetylcholine on oocyte maturation, this implies that cholinergic signaling might be related to DNA amplification in the oocytes. One putative result of such induction could be cholinesterase gene amplification, which in turn might protect the cells in which it occurs from the inhibitory effects of exposure to organophosphorous poisons. In other terms, one may perceive the changes in acetylcholine concentration as an alarm system and the amplification of cholinesterase genes as a defense mechanism against environmental intoxication.

Prospects for Future Research

In addition to the amplification of genes encoding target proteins for cytotoxic inhibitors and of oncogenes enhancing growth and cell division processes, gene amplification also appears to be a normal process in the development of various organisms. A well-known example is the amplification of chorion genes in insect larvae (40). To the best of our knowledge, there are as yet no parallel examples in mammals. However, the amplification and overexpression of the N-MYC oncogene in neuroblastoma have been proposed to represent a normal embryonic process, reflecting the undifferentiated nature of these tumor cells (41). It would be interesting to examine whether the cholinesterase genes are similarly subjected to such amplification during germline cells development and embryogenesis.

Amplified and overexpressed cholinesterase genes might enhance the rate of tumor cell growth and division in a yet unexplained mechanism. In this case, this phenomenon should be related with relapse and/or progress of disease and could be considered as a prognostic/diagnostic measure or as a follow-up marker for particular tumors. Further future directions for the investigation of cholinesterase gene amplification could be focused on efforts to block the expression of CHE and ACHEmRNA transcripts, for example, by "antisense" oligodeoxynucleotides (42). This may lead to the development of a therapeutic treatment blocking the growth-related effects of cholinesterases. Several other directions exist for studying the amplification of human cholinesterase genes, an intriguing phenomenon with multiple basic and applied implications, and will depend largely on future observations.

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