Cancer Therapy & Oncology
International Journal
ISSN: 2473-554X

Juniper Publishers
key to the Researchers

Review Article
Volume 6 Issue 5 - September 2017
DOI: 10.19080/CTOIJ.2017.06.555700

Canc Therapy & Oncol Int J

The Third Domain Ligand Binding Fragment of Alpha-fetoprotein: Detection of Metastasis-associated Molecular Targets

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Submission: August 31, 2017; **Published:** September 11, 2017

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Abstract

The vast majority of cancer deaths are due to metastasis to distal sites rather than from the primary tumor mass. Previous reports have established that the third domain of the alpha-fetoprotein polypeptide (AFP3D) consists of amino acid sequence stretches that participate in protein-to-protein docking interactions. These were detected by means of a computer software program configured to recognize and localize peptide sequences on specifically targeted proteins. The present report identified sequence fragments on AFP3D that could potentially interact with multiple metastasis-related proteins. The metastatic proteins were derived from four classes of such candidates, namely:

a. Cell adhesion and cell-to-cell contact proteins;

b. Extracellular matrix proteins;

c. Growth factor receptors; and

d. Growth factors and kinase enzymes.

Following detection and identification of the AFP3D-to-metastatic protein interaction sites, the "in silico" localized amino acid sequences were compared and aligned with prior published AFP interaction sites comprising hydrophobic ligands, various receptors, cell cycle proteins, and cation channels. Attempts were then made to assess the potential of multiple ligands/proteins competing for interaction sites on AFP3D. Previous publications and the present report have confirmed and verified the "in silico" identified interaction sites by experimental cell-based assays, mRNA microarrays, and kinase profile screenings. The experimentally-confirmed procedures and assays were performed in both in vitro MCF-7 cell cultures assays and in rodent animal models. Finally, the experimental findings on the AFP3D interactions with metastatic proteins were consistent with the computer-derived data.

Keywords: Alpha-fetoprotein; Growth factors; Cell adhesion; Receptors; Extracellular matrix; kinases; Cell contact; Metastasis

Abbreviations: AA: Amino Acid; AFP: Alpha-fetoprotein; 3D: Third Domain; PI3K: Phosphoinositol Kinase; mTOR: Mechanistic Target of Rapamycin; GAAD153: Growth Arrest and DNA Damage-Inducible-protein-153; PTEN: Phosphatase and Tensin Homolog; CHK: Checkpoint; GIP: Growth Inhibitory Peptide; CdK: Cyclin Dependent Kinase; PKC: Protein Kinase-C; MAP: Metastasis-associated Proteins; ECM: Extracellular Matric; MMP: Matrix Metalloproteinase's; ADAM: A Disintegrin and Metalloproteinase; KISSR: Kisspeptin Receptor; TSHR: Thyroid-Stimulating Hormone Receptor; LAMR: Laminin Receptor; TGF: Transforming Growth Factor

Introduction

Historical Background

Human alpha-fetoprotein (AFP) was one of the first biomarkers developed as a cancer biomarker and is still today considered the "gold standard" biomarker for liver cancer. AFP is a glycopolypeptide exhibiting a molecular mass of 69 kD with a 3-5% carbohydrate content [1]. Structurally, the full-length AFP molecule consists of three domains forming nine layers of peptide loops configured by 15 cysteine disulfide bridges [2]. The two-dimensional (secondary) image of AFP has been determined and been confirmed to be a V- or U-shaped molecular structure. Human AFP is classified as a member of the albuminoid gene family which consists of albumin, alpha-albumin, AFP, vitamin-D binding protein, and the AFP-related gene (ARG) protein [3].

Physiologically, AFP is known to be a serum carrier/transport molecule for fatty acids, bilirubin, retinoids, steroids, flavonoids, phytoestrogens, organic dyes, and various drugs [4]. This fetal glycoprotein can further serve as a regulator of both growth enhancement and inhibition. AFP has been denoted as an "oncofetal protein" being present during fetal development as well as adult tumorigenesis [5]. Hence, AFP has served both as a tumor and fetal defect biomarker in the clinical setting.

The existence of AFP receptors, binding, and interacting proteins have been reported for more than 25 years. It is now widely acknowledged that AFP binds to multiple cell surface receptors and cytoplasmic binding proteins [6-8]. However, AFP has never been reported to enter the intact nucleus, but is known to reside in the perinuclear space compartment following synthesis and/or cell uptake. Recent studies, reviews, and updates have established that the third domain of AFP is a multi-ligand binding fragment capable of binding and interacting with a vast array of steroids, retinoids, fatty acids, drugs, growth factors, and various protein and peptide receptors [9,10]. At least three major groups of cell surface receptors are known to bind AFP, namely:

i. The scavenger;

ii. Mucin; and

iii. Chemokine receptors.

The intracytoplasmic binding proteins interacting with AFP include the retinoic acid receptor, caspase-3, nuclear steroid receptors, phosphatase and tensin homolog (PTEN), and the growth arrest and DNA damage inducible protein 153(GADD153) [11]. It is further conceivable that AFP could interact with nuclear proteins during mitosis when the nuclear envelope temporarily dissolves. Other potential cell proteins interacting with AFP could include cell cycle proteins, lysolipid receptors, and cation channel proteins [12].

Data on mapping of protein-to-protein interaction sites on the third domain fragment of AFP (AFP3D) has been generated by means of computer" in silico" software. Many, if not most, of the computer-modeling protein interaction sites on AFP3D being reported have already been experimentally confirmed and verified in cell-based assays [12,13]. The present report describes a further type of in silico protein interaction with AFP3D, namely, metastasis-associated proteins such as:

a. Cell adhesion,

b. Extracellular matrix (ECM) proteins,

c. Growth factor receptors, and

d. Growth factor and kinase proteins.

Their cell functions encompass activities such as homophilic cell-to-cell adhesions, calcium-dependent stabilizing and maintaining cell connections, cell gap junction establishment, cell migration, anchorage-dependent cell death (anoikis), tumor invasion, degrading of extracellular matrix (ECM), integrin interaction, basement membrane adhesion and degradation, collagen proteolysis, and others (Table 1). The association of AFP with metastasis-associated proteins has not been previously reported, pursued, or addressed in the biomedical literature even though AFP has long been known to enhance metastasis in liver and other AFP-secreting tumors.

The continued search of new receptor and protein interacting sites on AFP3D can be scientifically justified in order to identify and provide insight into new and novel activation sites, receptor docking and occupation sequences, ligand targeting, and signal transduction networks. Such novel findings could pave the way for utilizing sub domains of AFP3D fragments as drug delivery platforms that target cells involved in cancer, autoimmune or inflammation disorders. Finally, new insights could be gained toward the identification of previously unknown binding/interaction partners for AFP that might enhance our understanding of AFP-to-target cells involved in the propagation of extra- and intracellular signaling.

Objectives and Aims

The objectives in the present report were to first pursue, search, and identify potential metastasis-associated protein interaction sites on AFP3D. Computer-modeling and protein-to-protein interaction and docking software were employed to identify specific amino acid (AA) sequence segments on AFP3D that could plausibly interact with known metastasis-associated proteins (MAPs) reported in the literature. Secondly, the different subtypes of MAPs are discussed in lieu of their specificity for engaging metastatic activation and/or deactivation processes and their possible interaction with AFP3D to alter or influence tumor cell detachment, spreading, and dissemination. Third, the MAP localization sites are compared to previously reported ligand and receptor binding sites already confirmed on AFP3D. Fourth, each member of the MAPs is discussed regarding its cellular activities and reported associations with ligand and receptor sites previously reported on AFP. Finally, experimental reports of AFP-derived peptide interaction with MAPs will be addressed in lieu of the present in silico findings.

Computer Modeling and Molecular Docking Software

The computer modeling of the 3D structure of human AFP was previously described [12-14]. Molecular docking (interaction) sites on AFP were identified and localized by means of proprietary computer software program (Peptimer Discovery Platform) developed and generously provided by Serometrix LLC, Syracuse, NY, as described in detail in prior reports [13,15,16]. The proprietary software modeling and simulation of protein-to-protein interaction sites are highly reproducible as described in previous research reports and have been repeatedly validated using in vitro whole cell-based assays and receptor binding measurements [17,18].

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Metastasis-Associated Proteins

Cell Adherence and Cell-to-Cell Contact Proteins

The total cadherin super family of proteins includes protocadherins, desmogleins, and desmocollins all of which function in cell adhesion and the formation of adherin junctions to link cells together [19]. The cadherins are dependent on calcium (Ca++) ions to function in cell-to-cell adhesion and these proteins contain extracellular Ca++ binding repeat domains and intracellular domains for adaptor and cell signaling functions. Different subtypes of cadherins can join cells together in both homotypic and heterotypic binding fashions and are classified depending on the cadherin prefix indicated (N = neural, E = epithelial, etc.) in their name [20].

Table 1a: The properties of the metastasis-associated proteins (MAPs) interacting with the third domain of AFP are listed.

I. Name of MAP Protein	NCBI Accession Number (#)	Amino Acid Length (AA)	Estimated Molecular Weight (D)	Function, Cell Type & Location	AFP Amino Acid (AA) Binding Sequence
1) Protocadherin Beta-1 (PCDH-β1) A member of family of cadherin-like cell homophilic adhesion proteins	Q9Y5F3	818	92,679	Calcium dependent cell adhesion protein involved in stabilizing & maintaining cell connections; active in neuron development	$_{401}$LQKYTQES $_{405}$IQESQALA $_{440}$QLTSSELM $_{500}$CTSSYANR $_{508}$RPCFSSLV $_{554}$QKLISKTR $_{569}$EAVIADFS
2) E-Cadherin-6 (ductal breast cancer) CDH6	AAH00019	663	75,118	Plays key role in Calcium dependent cell adhesion; binds cells together	$_{385}$FQTENPLE $_{500}$CTSSYANR
3) Cadherin-13 (CDH13)	NP_001248	713	80,783	Cell surface protein involved in Axon growth; protects endothelial cells from oxidative stress	$_{401}$LQKYIQES $_{421}$KLGEYYLQ $_{441}$QLTSSELM $_{500}$CTSSYANR
4) Cadherin-22 (CDH22)	CAB51587	828	93,812	Cell-to-Cell adhesion shows affinity to p120 catenin to stabilize E-cadherin at the cell membrane	$_{449}$AITRKMAA
5) Contactin associated protein-3 (CNTNAP2)	NP_387504	1288	145,931	Functions as a cell adhesion molecule or receptor. Member of Neurexin family associated with potassium channels	$_{481}$LGHLCIRH $_{516}$VDETYVPP $_{548}$KQEFLINL $_{569}$EAVIADES
6) Neural Cell Adhesion Molecule-1 (NCAM) (CD56)	NP_000606	848	96,078	A homophilic binding glycoprotein for cell-to-cell adhesion, and neurite outgrowths	$_{433}$VAYTKKAP $_{508}$RPCFSSLV
7) Platelet Endothelial Cell Adhesion Molecule (CD31) (PECAM-1)	NP_000433	738	83,615	PECAM-1 plays a key role in removing aged neutrophils from the body. Present in intracellular junctions, IgG family	$_{409}$QALAKRSC $_{441}$QLTSSELM
8) Connexin GJA5 (the A family of GAP junction proteins	AAH13313	358	40,561	A transmembrane gap junction protein, essential for cardiac muscle, embryonic development: maintains the microvasculature	$_{413}$KRSCGLFQ $_{529}$DKFIFHKD $_{533}$FHKDLCQA $_{558}$VKQKPQLT
9) Neurotropic Tyrosine Receptor-3 (NT-3)	NP_001012338	839	95,059	Promotes cell migration & invasion, KRAS signaling, aids in cell survival and differentiation, and transmembrane activity	$_{421}$KLGEYYLQ $_{441}$QLTSSELM $_{461}$CCQLSEDK $_{529}$DKFIFHKD

Group # I: Name: Cell Adherence and Cell-to-Cell Contact.

AA: Amino acid; KRAS: Cell Division Regulator Enzyme.

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Table 1b: The properties of the metastasis associated proteins (MAP) interacting with the third domain of AFP are listed.

I. Name of MAP Protein	NCBI Accession Number (#)	Amino Acid Length (AA)	Estimated Molecular Weight (D)	Function, Cell Type & Location	AFP Amino Acid (AA) Binding Sequence
1) Matrix metallo-protease-2 (MMP-2)	ABD38929	660	74,778	Enzyme involvement in blood vascular re-modeling, angiogenesis, tissue repair, tumor invasion, degrading ECM proteins	$_{444}$SELMAITR
2) Matrix-metallo-protease-9 (gelatinase-B) (MMP-9)	CAC10459	707	80,103	Enzyme that degrades ECM proteins, cell migration, metastasis, angiogenesis, wound healing, reproduction, and bone development	$_{409}$QALAKRSC $_{413}$KRSCGLFQ $_{444}$SELMAITR $_{449}$AITRKMAA
3) Matrix-metallo-protease-10 (Transin-2) (MMP-10)	AAH02591	476	53,931	Enzyme that degrades ECM proteins, role in development, morphogenesis, cell fate, acts on cell surface molecules, metastasis, hemopexin binding	$_{433}$VAYTKKAP $_{436}$KKAPQLTS $_{449}$AITRKMAA $_{453}$KMAATAAT $_{497}$VGQCCTSS
4) Matrix Metallo-Protease-13 (MMP-13) (Collagenase-3)	AAH74808	471	53,364	Collagen degrade, collagen re-structure, metastasis, reproduction, embryonic development, bone mineralization	$_{409}$QALAKRSC $_{449}$AITRKMAA $_{477}$ADIIIGHL $_{485}$CIRHEMTP $_{525}$AFSDDKFI $_{557}$VQKPQIT
5) Disintegrin & Metallo-Protease-22 (ADAM-22) (zinc protease)	NP_068368	899	101,857	Related to snake venoms, cell-to-cell & cell to matrix interactions, neurogenesis, fertilization, cell adhesion/migration	$_{429}$NAFLVAYT $_{433}$VAYTKKAP $_{481}$IGHLCIRH $_{512}$SSLVVDET
6) Integrin Alpha-2 (ITGA2) (Integrin α2/β1 Complex) (CD49β) (VLA-2α)	NP_002194	1181	133,807	Integrin-α2 links to integrin-β1. It's a receptor for laminin, collagen, fibronectin and E-Cadherin. Adhesion of platelets to collagen, organizes synthesized ECM proteins, collagen gene expression, metals	$_{409}$QALAKRSC $_{433}$VAYTKKAP $_{444}$SELMAITR $_{481}$IGHLCIRH $_{485}$CIRHEMTP $_{497}$NGQSSTSS $_{504}$YANRRPCF $_{508}$RPCFSSLV $_{522}$LINLVKQK $_{529}$DKFIFHKD $_{548}$KQEFLINL
7) Integrin Alpha-6 (IGA-6) (VLA-6)	AAH50585	686	77,724	Integrin – alpha6 links to various β-chains, cell adhesion, cell surface signaling, cell migration	$_{413}$KRSC6LFQ $_{436}$KKAPQLTS $_{444}$SELMAITR $_{485}$CIRHEMTP $_{525}$AFSDDKFI

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8) ANNEXIN-A8 Anti-Coagulant (ANXA8L2)	P13928	327	37,049	A conserved Calcium phospholipid binding protein: inhibits thromboplastin complex	$_{504}$ANRRPCF $_{512}$SSLVVDET
9) Collagen Type IV, Alpha-3	CAA56335 CAI17003	1670	188,078	Major component of basement membranes, multimeric compound that is composed of 3 subunits	$_{545}$LQTMKQEF $_{597}$QKLISKTR

Group # II: Name: Extra-cellular Matrix Proteins.

AA: Amino Acid; ECM: Extra-Cellular Matrix.

Table 1c: The properties of the metastasis associated proteins (MAP) interacting with the third domain of AFP are listed.

I. Name of MAP Protein	NCBI Accession Number (#)	Amino Acid Length (AA)	Estimated Molecular Weight (D)	Function, Cell Type & Location	AFP Amino Acid (AA) Binding Sequence
1) G-Protein Coupled Receptor-54 (GPR54) (KISS1R)	AAK83235 QT69F8 NP-115940	398	45,093	Serves as receptor for metastin (Kiss peptin-54), induces cell cycle arrest, a metastasis suppressor protein, GNRH*, Hypothalamus	$_{481}$IGHLCIRH $_{560}$CTSSYANR
2) Interleukin-8 Receptor (IL-8R) CXCR2	NP_001548	360	40,788	Chemokine Receptor-2 for interleukin-8, mediates neutrophile migration sites of inflammation, angiogenesis	$_{429}$NAFLVAYT $_{444}$SELMAITR $_{522}$LINLVKQK
3) fms-like Tyrosine Kinase Receptor	AAB23636	1298	147,064	Disables proteins causing blood vessel growth, serves as a receptor for VEGF, a soluble protein receptor	$_{421}$KLGEYYLQ $_{522}$LINLVQK $_{569}$EAVIADFS
4) Fibrablast Growth Factor Receptor-4 (FGFR4)	P22455 AAB25788	802	90,866	Regulates cell growth & proliferation, cell type, blood vessel formation, angiogenesis	$_{401}$LQKYIQES $_{477}$ADIIIGHL $_{504}$YANRRPCF
5) Laminin Receptor (67Kd) LAMR	AAC50652	295	33,423	Cell adhesion to basement membrane and signaling transduction	$_{508}$RPCFSSLV $_{522}$LINLVKQK
6) Thyrotrophin (TSH) Receptor	AAD31568	174	16,655	Stimulates production of thyroxine. G-protein coupled membrane receptor	$_{440}$QLTSSELM $_{597}$QKISKTR
7) Somatostatin Receptor-2	NP_001041	369	41,807	Suppresses retease of hormones, proteins	$_{512}$SSLVVDET
8) Ephrin Receptor 2β EPHR	CAI22899	986	111,714	A receptor kinase membrane-bound, concerning cell migration, tissue boundaries, axon guidance, cell segmentation, angiogenesis	$_{453}$KMAATAAT $_{477}$ADIIIGHL $_{508}$RPCFSSLV
9) Met Oncogene Hepatocyte Growth Factor Receptor (C-Met)	NP_000236	1390	157, 487	Tyrosine Kinase Enzyme, single transmembrane receptor, C-Met triggers growth, angiogenesis, and oncogenesis	$_{441}$SELMAITR $_{481}$IGHLCIRH

Group # III: Name: Growth Factor Receptors.

*GNRH: Gonadotrophia Releasing hormone; AA: Amino Acid.

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Table 1d: The properties of the metastasis associated proteins (MAP) interacting with the third domain of AFP are listed.

I. Name of MAP Protein	NCBI Accession Number (#)	Amino Acid Length (AA)	Estimated Molecular Weight (D)	Function, Cell Type & Location	AFP Amino Acid (AA) Binding Sequence
1) Transforming Growth Factor-Beta-1 (TGF-β)	NP_000651	390	44,187	A polypeptide growth factor for cell growth, proliferation, differentiation, and apoptosis, Induces Transformation	$_{413}$KRSCGLFQ
2) Vascular Endothelial Growth Factor (VEGF)	CAC19516	371	42,034	Stimulates vascular permeability and vasculagenesis, angiogenesis, restores 0_2 Supply	$_{477}$ADIIIGHL $_{497}$QKLISKTR
3) Retino-Blastoma – Associated (E2F) Protein-1 (RBA1)	NP_000312	928	105,142	DNA binding transcription factor regulating cell cycle, a tumor suppressor, promotes G0 to G1 cycle transition	$_{560}$CTSSYANR
4) p53 Protein Cellular Tumor Antigen (Phosphoprotein-53)	NP_000537	393	44,527	Prevents cancer formation, tumor suppressor, preserves genome stability, binds DNA and regulates gene expression, prevents mutation	$_{429}$MAFLVAYT $_{453}$KMAATAAT $_{477}$ADIIIGHL $_{516}$VDETYVPP $_{522}$LINLVKQK $_{525}$AFSDDKFI $_{529}$DKFIFHKD
5) Tyrosine Protein Phosphates Non-Receptor Type-7 (PTP)	NP_002823	465	52, 684	PTPs regulate cell growth, differentiation mitotic cycle, and oncogenic transformation	$_{421}$KLGEYYLQ $_{440}$QLTSSELM $_{477}$ADIIIGHL $_{489}$EMTPVNPG
6) C-Terminal Binding (CTB) Protein (Transcriptional Regulator)	AAD14597 AAH7021	440	49,852	Transcriptional suppressor enzyme regulates cell growth, migration, immune response, differentiation	$_{391}$LEKCFQTE $_{401}$IQESQALA $_{477}$ADIIIGHL $_{533}$FHKDLCQA $_{560}$CTSSYANR
7) NME1 Nucleoside Di-Phosphate Kinase (NDPK)	CAG46912	152	17,221	Regulates NF-kβ Signaling and catalzes phosphorylation of nucleotide monophosphates to diphosphates	$_{477}$ADIIIGHL
8) Metastasis Suppressor Protein (MTSS1)	AAF15947 NP_055566	755	85,541	Serves as a tumor metastasis suppressor, acts with actin cytoskeleton in multiple organ sites	$_{425}$YYLQNAFL $_{444}$SELMAITR

Group # IV: Name: Growth Factors/Regulators.

AA: Amino Acid; NF-kβ: Nuclear Factor B-cell activator.

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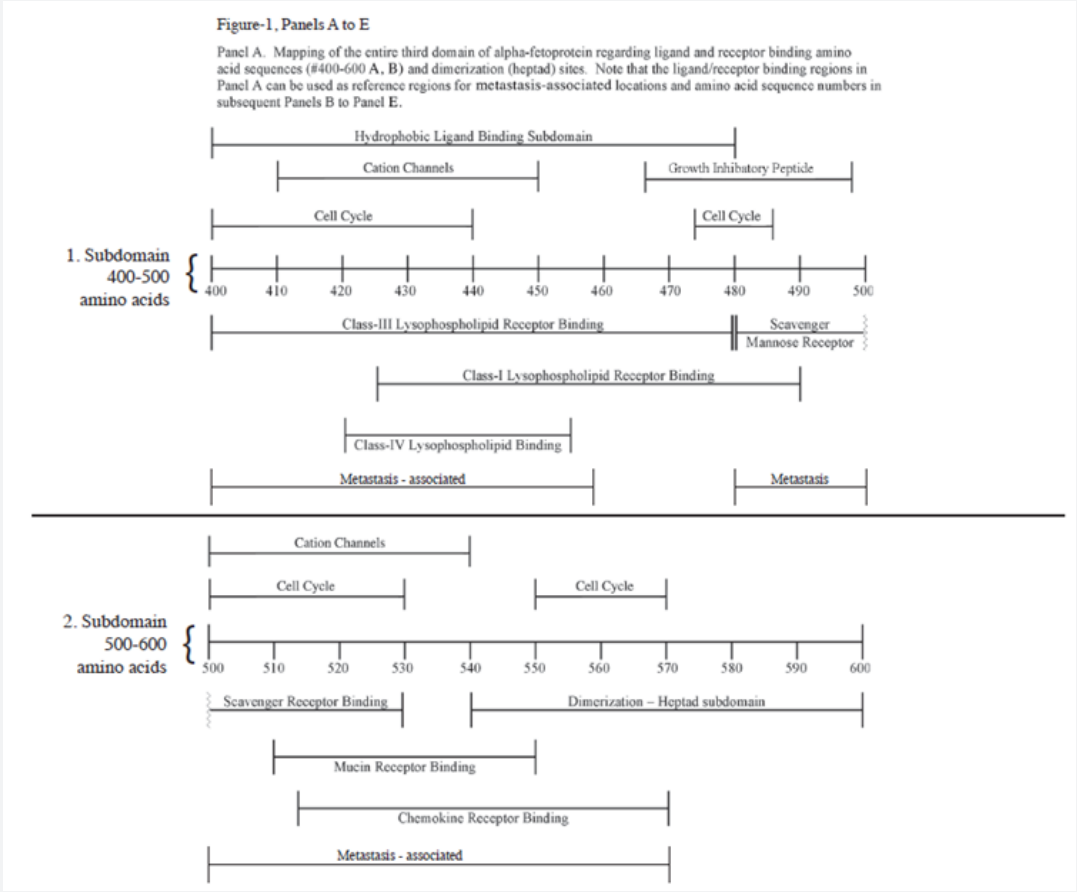

Figure 1A: Panel A- Mapping of the entire third domain of alpha-fetoprotein regarding ligand and receptor binding amino acid sequences and dimerization sites.

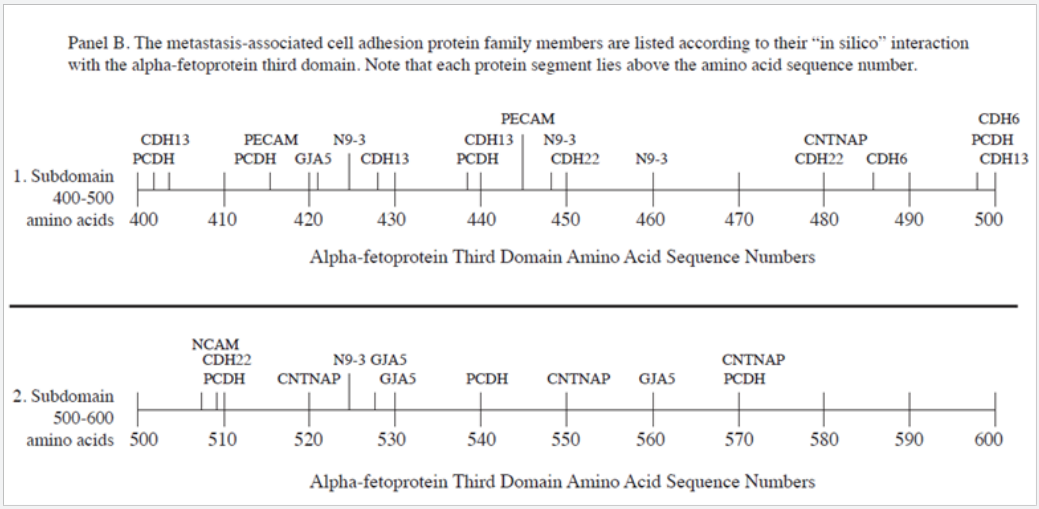

Figure 1B: Panel B-The metastasis-associated cell adhesion protein family members are listed according to their in silico interaction with the alpha-fetoprotein third domain.

The cadherins that interact in silico with AFP3D are listed in (Tables 1a-1d), Group 1 and Figures 1A & 1B and included protocadherin β 1 (neural development), E-cadherin-6 (ductal epithelium), cadherin-13 (endothelium) and cadherin-22 (epithelial). Contactin associated protein-3 was also found; such proteins function in concert with potassium channels and are sub-members of the Neurexin family [21]. The neural cell adhesion molecule-1 interaction with AFP also occurred and this protein functions in hemophilic cell-to-cell contact in neurite outgrowths [22]. The platelet endothelial cell adhesion molecule

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(CD31) also interacts with AFP3D and functions to remove aged white blood cells (i.e. neutrophils) from the bloodstream and intracellular tissue spaces [23]. Another AFP3D interacting adhesion protein found was connexin which plays a role in cell gap junction formation in vascular and heart tissue during embryo/fetal development [24]. Finally, a neurotrophic tyrosine receptor 3 sites was found which is known to function in cell migration following KRAS signaling and cell transmembrane activities (Table 1).

Extracellular Matrix (ECM) Proteins

The EMC proteins encompass the families of zinc matrix metalloproteinases (MMP), integrins, a distintegrin and metalloproteinase (ADAMS), collagenases and gelatinases, and annexins [25]. The MMPs are secreted as inactive pro-forms which are cleaved to produce a catalytic hemopexin domain which participates in collagen degradation. The MMPs are involved in the breakdown of the ECM in physiological events, development, disease, and metastasis. The ADAMS family members are membrane-anchored proteins structurally related to snake venom disintegrins being implicated in cell migration, metastasis, cell adhesion, and cell-to-cell contact during neurogenesis, fertilization, reproduction, muscle development, and integrin binding [26]. The collagenases and gelatinases are sub members of the MMP family involved in the breakdown of ECM proteins for skeletal bone mineralization, tissue remodeling, reproduction, and in diseases such as rheumatoid arthritis and osteoarthritis [27]. Integrins are cell surface transmembrane proteins, consisting of α / β chains, that participate in cell adhesion and cell membrane mediated signaling [28]. Finally, the annexins are Ca++ and phospholipids' binding proteins which function in the blood coagulation cascade [29].

Table 2: Global RNA microarray data: Transcripts displaying 1.0 or larger log fold (log base 2.0) decrease (down-regulation) for genes associated with cell adherence, ECM, and metastasis obtained from Human MCF-7 breast cancer cells *in vitro**. Data was obtained by employing AFP 3rd domain-derived peptides [79].

Gene/Protein NAME	Fold Log 2 Decrease (-)	Cell Function Related to Metastasis
1. Adam – 2.2	-5.0	Cell to ECM contact
2. Collagen - IV	-4.1	ECM-basement membrane
3. p53 Apoptosis Inducing Factor	-3.3	Cancer Suppressor
4. Cadherin-13 (CDH13)	-2.3	Nerve Cell Growth
5. Contactin-associated – 3)	-1.6	Adhesion molecule
6. Claudin – 13	-1.5	Cell Junctions
7. Neural Cell Adhesion Molecule	-1.4	Cellular Adhesion
8. Connexin-40 GAP	-1.3	Cell GAP Junctions
9. Protocadherin – β1	-1.1	Ca++ cell adhesion
10. COLLAGEN XI, α2	-0.8	Tissue Support Structures
11. Platelet Endothelial Adhesion	-0.8	Intracellular Junctions
12. MTSS Metastasis Suppressor	-0.8	Tumor Suppressor
13. Integrin – alpha-2	-0.5	Adhesion Receptor
14. Cadherin – 22	-0.5	Cellular Adhesion
15. Annexin – A8	-0.3	Anti-coagulant Factor
II. Growth Factor & Kinases		
1. Nucleoside-diphorylate Kinase	-4.1	Regulates NFkB signals
2. Neuotropic Ser/THr Kinase	-3.2	Promotes Cell Migration
3. Ser/THr Kinase-33 (STK33)	-3.2	Cell migration associated
4. Ephrin Receptor Kinase	-1.3	Regulates cell migration
5. GSK Kinase - 3α	-0.6	Cell Growth/migration
6. SEMA Domain (Ig) TyR Kinase	-0.6	Cell Guidance Signals
7. NFkB related Kinose	-0.4	Controls cell survival
8. L-1 Receptor associated Kinase-1	-0.4	Up regulates NFkB

*Expression of 716 transcripts was significantly altered in MCF-7 cells after 8 days of treatment with AFP – derived peptides as compared to treatment with the scrambled peptide. Four hundred thirty RNAs were down regulated, while 286 RNAs were up regulated. Data provided in collaboration with Dr. Kathleen Arcaro, University of Massachusetts, Amherst, MA [32,33]. ECM: Extracellular Matrix.

The specific metastasis-associated ECM proteins interacting with AFP3D in silico are shown in Figure 1C and Table 2, Group 2. The MMP family members which were detected included MMP-2, MMP-9, MMP-10, and MMP-13 [25]. While MP-2 functions in angiogenesis, tissue repair, and tumor invasion, MMP-9, MMP-10, and MMP-13 are largely involved with ECM degradation, remodeling, and metastasis. AFP3D was also found to react with ADAM-22 which has been implicated with cell migration, steroid

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responsiveness, myelin and brain development, and cell-to-cell interactions [26]. The integrins interacting with AFP3D included both integrin alpha-2 and integrin alpha-6. Integrin alpha-2 (ITGA2) serves as a receptor for laminin, collagen, collagen C-propeptides, fibronectin, and E-cadherin. ITGA2 is responsible for platelet and multiple cell adhesions utilized in for the tissue organization of collagen and other ECM proteins [29]. Integrin alpha-6 (ITGA2) also interacted with AFP3D in silico; ITGA2 (also known as VLA-6) combines with other integrin members to form heteropartner complexes. These partners participate in both cell adhesion and in cell surface-to-cytoplasmic signal transduction. The annexins are calcium and phospholipid binding proteins which inhibit the formation of the thromboplastin-specific complexes in clot formation [29]. Finally, the collagen type IV alpha-3 protein adds structure and support to tissues such as bone, cartilage, eye, and muscles [30].

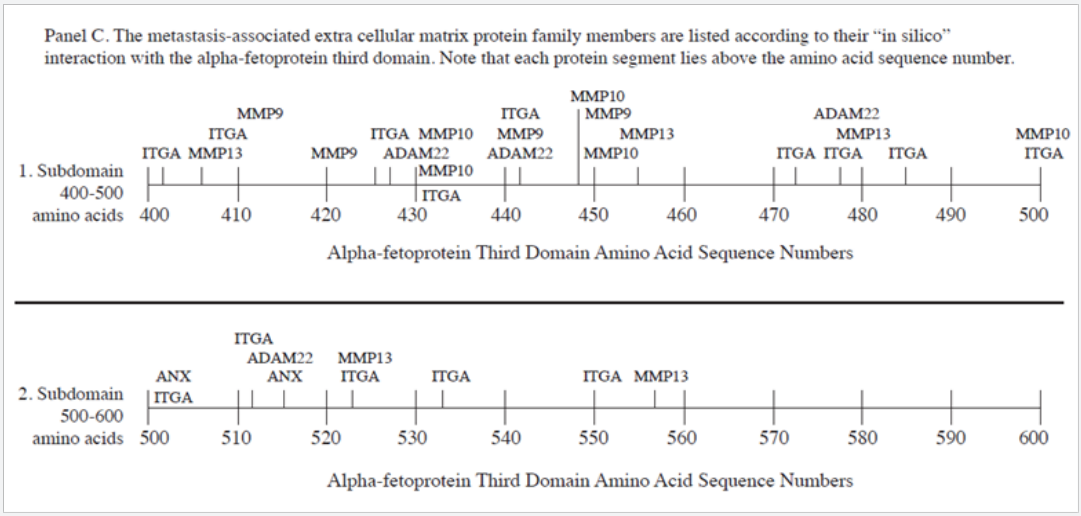

Figure 1C: Panel C- The Metastasis associated extra cellular matrix protein family members are listed according to their "in silico" interaction with the alpha-fetoprotein third domain.

The Growth Factor Receptors

The metastasis-associated growth factor receptors (GFRs) include a diverse array of receptor proteins encompassing biological processes such as angiogenesis, growth enhancement and inhibition, kinase activity, mitogenesis, differentiation, and cancer tumorigenesis [31]. Many, if not most, are G-coupled seven-transmembrane receptors with various kinase domains involving intracellular signaling and direct cell-to-cell contact interactions. Their cell surface signaling sets in motion a cascade of downstream signals which ultimately influence, regulate, or mitigate mitogenesis, cell invasion and migration, and metastasis. Some receptors containing tyrosine kinases can serve to activate and/or disable proteins (fMS-like) that cause growth in a variety of tissues including blood vessels [32]. Other such receptors (somatostatin receptor) can inhibit release of many types of hormones and secretory proteins [33]. For example, the ephrin receptor is involved in multiple regulation activities in tissue formation, cell migration, axon guidance, cell-to-cell interactions, stem cell differentiation, and cancer growth. Furthermore, the G-coupled receptor-54 serves as a receptor for the KISS gene product, a metastasis suppressor protein that inhibits metastasis, invasion, and chemotaxis of melanomas and breast cancer [34]. In summary, metastasis-associated growth factor receptors are intimately involved with cell-matrix adhesion, cell invasion and migration, angiogenesis, and cancer growth.

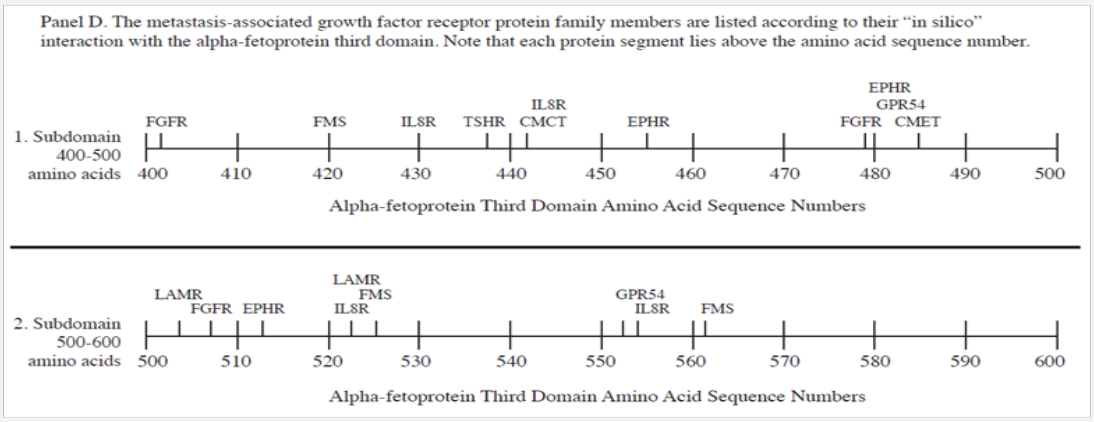

Figure 1D: Panel D- The metastasis-associated growth factor receptor protein family members are listed according to their "in silico" interaction with the alpha-fetoprotein third domain.

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The metastasis-associated growth factor receptors interacting with AFP3D in silico are presented in Figure 1D and in Table 2, Group 3. The GFR group members detected were comprised of G-coupled receptors and other transmembrane proteins such as GPR54 (KISSR), interleukin-8 receptor (IL-8R), fMS-like tyrosine receptor (fMS-KR), fibroblast growth factor receptor (GFGR4), laminin receptor (LAMR), thyrotrophin receptor (TSHR), somatostatin receptor-2 (SR2), ephrin receptor (EPHR), and c-Met receptor. The KISSR, which is known to interact with AFP3D serves to regulate the KISS tumor suppressor by inhibiting cell proliferation, cell migration, and tumor metastasis [34]. The second GFR to interact with AFP3D was found to be IL-8R which binds IL-8 with high affinity and transduces a signal through the G-protein activated second messenger pathway [35]. IL-8R can inhibit embryonic oligodendrocyte precursor cell migration in the developing spinal cord and is involved with neutrophil chemotaxis activation. The fMS-KR is a soluble tyrosine kinase enzyme that serves as a receptor of vascular endothelial growth factor (VEGF), a potent angiogenic growth factor; fMS-KR serves to reduce free circulating levels of VEGF [36].

The FGFR4 receptor, which interacts with AFP3D in silico, is known to bind to and interact with fibroblast growth factor to initiate cell growth and proliferation [37]. FGFR is over expressed in breast and ovarian cancer and other gynecological tumors. The LAMR that interacts with AFP3D is a 37 kD receptor precursor that forms complexes with p40 ribosome chromosome associated protein [38]. LAMR is a multi-functional metastasis-associated protein involved with cell adhesion, invasion and migration. The TSHR is located on thyroid cells and binds the thyroid-stimulating trophic hormone secreted by the pituitary. It stimulates production of thyroid hormone which regulates overall body metabolism [39]. The EPHR is the receptor for the Ephrin protein, serving as a receptor tyrosine kinase to enact cell migration and guidance functions especially in axon growth cones of the brain [40]. Ephrin signaling is also a factor in angiogenesis, stem cell development, and growth of tumors. Finally, the C-met oncogene product is a hepatocyte growth factor receptor tyrosine kinase. C-met receptor plays a role in cell survival, embryogenesis, and cell migration and invasion [41].

Growth Factors and Kinases

The metastasis-associated growth factors (GF) and kinases include multiple and diverse types of kinases involved in angiogenesis, vasculogenesis, oxygen supply to tissues, metastasis suppression, tumor formation, genome mutation, di- and tri-phosphate chemical exchange cell growth and proliferation, development, signal transduction, and cell cycle phase transition. The GFs and kinases localized on AFP3D sequences include transforming growth factor-β 1 (TGF β 1), vascular growth factor (VEGF), retinoblastoma-associated E2F protein-1 (RBA1), p53 phosphoprotein-1 (TB53), tyrosine protein phosphatase type 7 (PTP7), c-terminal binding protein (CTBP), nucleoside di-diphosphate kinase (NDPK), and metastasis suppressor protein (MTSS1). Most of these proteins serve as tyrosine kinase enzymes and are involved in blood vessel growth and cell cycle regulation.

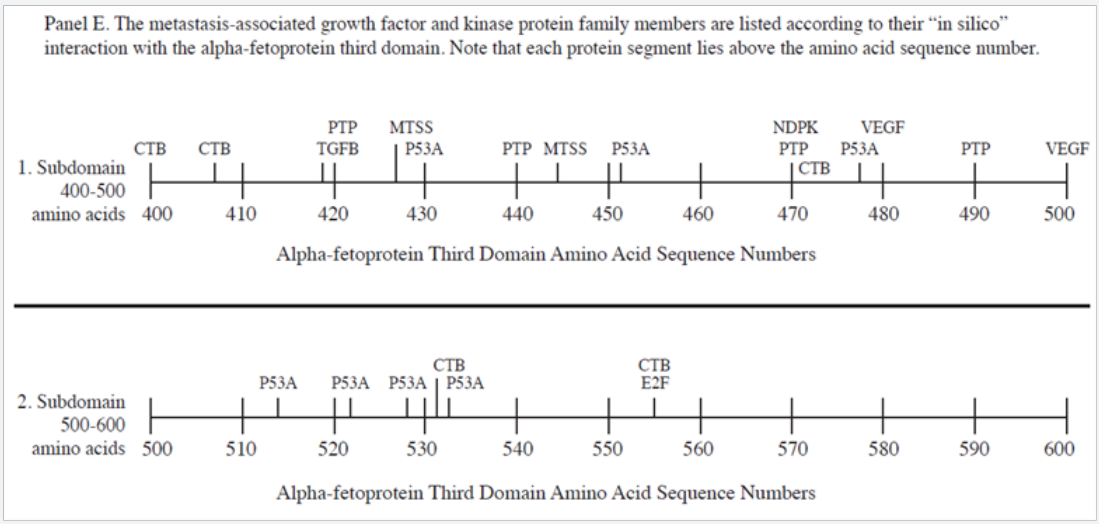

Figure 1E: Panel E- The metastasis-associated growth factor and kinase protein family members are listed "in silico" interaction with the alpha-fetoprotein third domain.

The metastasis-associated growth factors and kinases that interacted with AFP3D in silico are displayed in Figure 1E and on Table 2, Group IV. The GF and kinase protein (i.e. TGF β 1) interaction sites on AFP3D reflect proteins that promote cell growth and proliferation, tumor cell transformation, activation of transcription factors, and regulation of gene expression. It can further activate factors such as interferon-γ, tumor necrosis factor-α and multiple cytokines. VEGF is a growth factor for blood vessel formation which regulates vascular permeability [42]. This GF can further serve to restore blood vessel injury and form new vessels to bypass blocked vasculature. The RBP1 is a retinoblastoma-related member of the E2F family of

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DNA-binding transcription factors, which act as key proteins regulating the cell cycle and tumor suppressor proteins [43]. The RBP-encoded E2Fs are also targets of transforming proteins of small DNA tumor viruses. The TBp53, a cellular tumor antigen, is a phosphoprotein which functions as a tumor suppressor protein providing protection to the genome [44]. Hence, it serves as a conserving stability agent of genes in preventing cancer cell formation. The tyrosine protein phosphatase non-receptor-7 (PTP7) is a signaling molecule that regulates multiple processes including cell growth, differentiation, the mitotic cycle, and cancer cell transformation [45].

PTP7 can interact with lymphokine secreting cells, hematopoietic cells, and exhibits MAP kinase activities. A further protein interacting with AFP3D is the C-terminal binding protein which functions as a regulatory tyrosine kinase [46]. This enzyme phosphorylates the C-terminal ends of Src-family kinases. The nucleotide-di-phosphate kinase (NDPK) that also interacted with AFP3D is an enzyme that catalyzes the exchange of terminal phosphates between different nucleoside di- and triphosphates. NDPK is involved with cell growth and proliferation, development, signal transduction, and G-protein coupled receptor activities. Finally, MTSS1 is a metastasis suppressor protein that interacted with the AFP3D fragment. This protein contains an acting binding segment and is present in various tissues and organs [47].

Reported Interactions of Metastasis-related with Hydrobolic Ligands and Various Receptors/Proteins

The co-localizations of hydrophobic ligands and various receptors/proteins are demonstrated in Figure 1, Panels A-E and have been reported in the literature as described below. The metastasis-related protein cluster site segments detected on AFP3D were identified by amino acid sequence numbers on the AFP polypeptide chain Figure 1A. In Figure 1A, the first metastasis-protein cluster is displayed for AFP3D amino acid (AA) #400-455; the second cluster was found on AA #480 to 500; and the third cluster ranged from AA #500-570 with outliers scattered throughout the third domain. Literature searches described herein revealed that these clustered domain segments may not be found there by mere chance. For example, the hydrophobic ligand binding sub domain in Figure 1A, extends and includes the entire AA lengths of the metastasis-related protein interaction segments on AFP3D. Interestingly, published reports have demonstrated that tumor invasion and metastases are clearly linked to activities of N-3 polyunsaturated fatty acids and estrogens [48-50]. These metastasis-to-hydrophobic linkages are known to involve cell adherence proteins such as cadherins, ADAM proteins, β -catenin, and others [51-53].

In other studies, reports of cation channel influence on metastasis have emerged. Such studies have implicated selective and non-selective cation channels such as transient receptor potential vallinoid (TRPV), voltage-gated sodium channels, ATP-release channels, and gap junctions involving connexin in breast, brain, pancreatic, and lymphoid tumors [54-57]. Additional reports have shown correlations between metastasis and cell cycle proteins such as β -catenin, ubiquitin ligases, F-box proteins, SKP2 proteins, and DNA-PK kinases [58-61]. Chemokine and scavenger receptors (SR) have also been associated with metastasis through proteins such as SR Class B type 1 Lysl oxidase (LOX), CXCR6, CXCL16, SR Class-A, and low-density lipoprotein receptor [62-65]. Lysophospholipid receptors are also associated with the process of metastasis through the sphingo-1-phophate receptor, VEGF receptor, lysophosphatidic acid receptors, Sp1 kinase, and G-coupled protein receptor-55 [66-68]. Finally, the mucins and their receptors have been linked to metastasis via N-cadherin, NCAM, mucin-1, mucin-2, mucin-6, mucin-16, and mucin-5AC [69-71]. In summary, it can be discerned that the ligand and receptor interaction sites on AFP3D clearly correlate in the literature with their respective sub domain localizations displayed in Figure 1A.

The Relationship of AFP3D-localized Amino Acid Segments with Metastasis

A literature survey in Pub Med and other search engines readily reveal that AFP and its derived peptides are directly involved with the metastatic process. AFP serum levels have long been associated as a biomarker for various metastatic tumors. Such cancers include hepatocellular carcinomas, germ cell tumors, and reproductive and gastrointestinal cancers. However, AFP is also a metastatic biomarker for tumors such as gastric cancer, breast tumors, mixed germ cell tumors such as seminomas, and neuroendocrine cancer [72-77]. Thus, full-length AFP has been shown to play a critical role in the metastatic spread of cancer cells.

Literature reports have also employed short peptides (8-34 amino acid) derived from the third domain of human AFP. These reports have experimentally demonstrated that AFP3D-derived peptides do in fact interact with cell adhesion, cell-to-cell contact, receptor, and kinase proteins that clearly influence and directly affect the metastatic process [76-80]. One such study with human follicular thyroid carcinoma (FTC) cells and the AFP-derived "Growth Inhibitory Peptide (GIP) directly demonstrated in vitro that GIP significantly inhibited cell migration and invasion in a dose-dependent fashion [78]. A second study reported that GIP suppressed the spread of tumor infiltrates and metastases in both human and mouse mammary cancers [76,77]. A third report showed that GIP interfered with cell-to-cell contact inhibition, blocked tumor cell adhesion to ECM proteins, and prevented platelet aggregation thus preventing tumor cell spreading/migration and attachment to anchorage surfaces [76].

Furthermore, the AFP-derived GIP peptide has been demonstrated to down-regulate proteins associated with the metastatic process using a mRNA global microarray [79]. As shown in Table 2 (Part I), GIP was found capable of down-regulating the mRNA of at least 15 different cell adherence

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and ECM proteins associated with metastasis; these included proteins such as ADAM-22, connexin, cadherins, contact in, collagen, and the MTSS metastasis suppressor. Moreover, the mRNA down-regulation (employing a log base 2) in the GIP study ranged downward from a -5.0 fold decrease to -0.3. In addition, AFP-derived GIP down-regulated the mRNA of metastatic growth factors and kinase from a -4.1 decrease to -0.4 (Table 2), Part II. Finally, an enzyme activity screen of the AFP-derived peptide (GIP) incubated with metastasis-related kinases representing activities of protein kinase-C, MAP kinases, apoptosis signal kinases, and others, showed significant inhibitions ranging from 45 to 18% (Table 3) Part I. Moderately enhanced kinase activities ranging from 33 to 18% were further displayed in enzymes representative of ephrin, c-Met, fibroblast growth factor and NFkB-related kinases (Table 3) Part II.

Table 3: The percent of metastasis-associated kinase activity following AFP-derived peptide treatment is listed below. The control assay was 100% and the inhibition or enhancement is listed as percent activity of the control. Data was confirmed in IC50 titration curves.

I. Kinase Enzyme Name	Type SRC 2,3	Inhibition Percent +SD	Activity
1) ASK-1	Ser/Thr	28 ±4	Apoptosis-related
2) GSK-3β	SER/Thr	25 ±1	Glycogen Synthase, Insulin
3) HCK	Tyr	33 ± 2	Chemokine related
4) MKK7B	Ser/Thr	18 ± 9	G2 → arrest
5) PCKα	Ser/Thr	23 ± 2	Phospholipids TRP-related
6) PKC Λ	Ser/Thr	21 ± 11	Phospholipids TRP-related
7) TBK1	Ser/Thr	45 ± 8	NFKβ-related
II. Kinase Enzyme Name	Type SRC 2,3	Enhancement Percent ± SD	Activity
1) EpHA4	Tyr	30 ± 10	Neurons, cell migration
2) EpHB4	Tyr	19 ± 2	Neurons, cell migration
3) FgR	Tyr	19 ± 1	Leukocyte migration, Toll-R
4) IKK-β	Ser/Thr	18 ± 3	NFK-β related
5) Met	Tyr	21 ± 0	Proto-ongene product
6) ZAP70	Ser/Thr	33 ± 2	NFKβ-related

*Ser/Thr: Serine/thyronine kinase; Tyr: Tyrosine kinase

c-Src: A Non-Receptor kinase protein of the ser/Thr or tyrosine type that phosphorylates these residues in other proteins. The kinase activity screen for AFP-3D peptides was performed via the commercial "kinase profiler" by the Upstate Biosignaling Corp., Dundee Technology Park, Dundee, United Kingdom

Conclusion

Many previous reports have now documented in both computer-based and experimentally-verified studies that the AFP3D is comprised of short amino acid sequence that can associate with hydrophobic ligands and receptor/proteins [11-15]. It is now known that AFP3D amino acid sequences are capable of interacting with fatty acids, steroids, retinoids, ligands, and with various proteins including scavenger, mucin, chemokine, cation channel receptors, and cell cycle proteins. From Figure 1, it can be deduced that these ligands, receptors, and proteins appear to occupy and/or overlap various amino acid sequence sites. It is tempting to speculate that these compounds could act in combination with, or in competition with each other. AFP binding at overlapping sites in the case of fatty acids, estrogens, and retinoids have long been known and reported [1,2]. Thus, it would not be unreasonable to assume that competition of proteins to occupy binding/docking sites on AFP3D might occur. Presently, the metastasis interaction sites detected on AFP3D could also be subject to interference or conformational changes by ligands/proteins previously localized to those same sites. The literature reviews stated in this report confirmed that multiple metastatic proteins can and do interact with many of the ligands/proteins listed above. Therefore, it is plausible that co-localization of metastatic proteins with the multiple other ligands/proteins described herein, may be physiologically relevant.

It was further shown and addressed in the present report that metastasis-related proteins are indeed involved with cell adherence, cell-to-cell contact, and the extracellular matrix. These associations indicate that growth factors, and their receptors and kinases are involved in order to achieve the completed process of tumor cell detachment. Once tumor cells are separated and disseminated from the tumor mass, the segregated tumor cells must migrate and digest their way through various ECM environments and basement membranes to gain entrance and transpassage across the endothelial cells into the lumen of the blood vasculature and lymphatic ducts. The extravasations of tumor cells to distant sites occur only after the loss of cell adhesion and release of protolytic enzymes to digest a tunnel through a myriad of tissue membrane barriers. After the tumor cells have egressed through the blood vessels, they can attach, bind, and cluster with circulating platelets to ensure a shielded passage while traveling through the blood vessels [81,82]. By means of the process of chemotaxis, tumor cells are

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attracted to distal sites of filtration organs such as liver, lungs, kidneys, and bone marrow.

The blood vessel or lymphatic transpassage is again repeated and the tumor cells finally become "nested" within the stromal tissue of the host organ [82]. It becomes obvious that AFP3D interactions with the metastatic proteins described in this report can play a pivotal role in the enhancement or inhibition of the multi-stage process of guiding metastatic cells to distal sites. As demonstrated in Tables 2 & 3, certain AFP-derived peptide segments are capable of up- and down-regulation of the mRNA of various metastatic proteins with the subsequent inhibition or enhancement of the relevant metastatic kinases.

Conflict of Interest

No US federal grants were used there are no known conflicts of interest in the preparation of this manuscript.

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How to cite this article: Mizejewski GJ. The Third Domain Ligand Binding Fragment of Alpha-fetoprotein: Detection of Metastasis-associated Molecular Targets. Canc Therapy & Oncol Int J. 2017; 6(5): 555700. DOI: 10.19080/CTOIJ.2017.06.555700.

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