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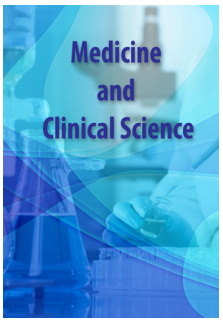

Borrelia Infected Neoplastic Glial Cells in Five Patients With Glioblastoma Multiformae

Alan B MacDonald

[1]*Molecular Interrogation Laboratory of Naples, Naples, Florida 34114, USA*

Correspondence

Alan B MacDonald, M.D.

Molecular Interrogation Laboratory of Naples, Naples, Florida 34114, USA

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Abstract

Glioblastoma multiformae (GBM) is a primary malignancy of brain astrocytes. Transcranial stereotactic biopsy specimens from five deceased and anonymous patients with GBM were retrieved from archival storage two years after the death of the patients. All patients were residents of the Long Island, New York metropolitan region where *borrelia burgdorferi* and *borrelia miyamotoi* infections are hyperendemic. Fluorescence in situ hybridization (FISH) using two DNA probes specific for genes of *burgdorferi borrelia* and an additional FISH method study with two DNA probes specific for *miyamotoi borrelia* each and individually produced high signal borrelia DNA hybridizations inside malignant cells in brain biopsies from all five decedents. Bright signal bacterial DNA hybridizations inside live borrelia spirochetes were found at all levels in serial sections of tumor biopsy fragments in low magnification imaging and outright invasion of the nuclei of individual tumor cells in high resolution imaging. Herein is the first report of a cluster of GBM patients with universal borrelia microbe invasion of neoplastic glial cells.

Introduction

Glioblastoma multiformae (GBM) is a malignant primary brain tumor of astrocytic glial cells. Fourteen peer reviewed and published manuscripts [1-14] have discussed the proclivities for borrelia spirochetes to adhere to human and mammalian glial cells in laboratory circumstances in vitro tissue culture studies. These precedents and epidemiology observations of apparent clusters of primary human brain malignancies in some patient cohorts with pre-existing chronic Lyme neuroborreliosis illnesses provided a conceptual model to explore the possibility of a role for a linkage of occult borrelia species brain infections and glioblastoma multiformae. Five such human glioblastoma deaths representing a geographic cluster of GBM patients from Suffolk County, New York, were previously presented in an unpublished research poster in 2016. (supplementary file) Biopsies of GBM from those five patients were studied in a Fluorescence in Situ Hybridization (FISH) method using *borrelia burgdorferi* specific DNA probes to detect bacterial DNA in sites of brain malignancy. Single *borrelia burgdorferi* spirochetes were visualized inside of all GBM tumors. Biofilm communities of borrelia spirochetes inside the GBM tumor biopsies were also present in all tumors. Biofilm type infections correlate with antecedent chronic infections . High signal FISH positive bacterial DNA hybridizations covered the entire surfaces of the GBM tumor biopsies. Bacterial DNA total content generally exceeded the total DNA content of the human DNA contained in the nuclei of malignant glial cells. These data strongly supported an association between simultaneous circumscribed borrelia glial cell infections of human brain and similarly sized circumscribed localizations of glial malignancy in biopsy tissue fragments. In 2022, a second round of FISH hybridizations utilized newly designed DNa probes to detect genes of a second New York endemic borrelia species , *borrelia miyamotoi. Miyamotoi borrelia* are members of the genetically distinct relapsing fever borrelia (RFB) group of species. A novel flagellin Gene (MiyaFla) DNa probe was designed to detect the *miyamotoi borrelia* gene for flagellin, which is genetically distinct from flagellin B gene of *burgdorferi borrelia*. In addition, a second DNa probe for the GLPQ gene of *miyamotoi borrelia* was designed. GLPQ genes are absent in all strains of *borrelia burgdorferi* and universally present in all *miyamotoi* and all relapsing fever borrelia (RFB). Neuroborreliosis illnesses caused by *borrelia miyamotoi* infections have been reported in deep brain sites presenting as non-malignant encephalomyelitis illnesses in Japanese patients and in patients from the Soviet Union. No GBM focused studies of

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human brain malignancies with miyamotoi infections have ever been reported. RFB borrelia infections in Africa have been circumstantially associated with some clusters of primary brain tumors. All patients in the present cohort were residents of a metropolitan New York region where *miyamotoi borrelia* infections and *burgdorferi borrelia i*nfections are co-vectored by Ixodes scapularis ticks.

Methods

Unstained histology slides were prepared from archival storage paraffin blocks of five anonymous decedent patients' stereotactic biopsies two years after their death. Approval for this retrospective was secured after institutional review by community hospital-based pathologists practicing in Suffolk County New York. A four-digit code number was inscribed on recut unstained slides representing ten levels of focal planes through each paraffin block containing the entire content of a series of stereotactic brain biopsies of each anonymous patient. All histological sections were cut at three-micron thickness in a paraffin ribbon, and heat annealed to clean glass slides. Strict patient confidentiality covenants were established by institutional guidelines for human research studies at the participating institutions. The only information released to this investigator was the pathological diagnosis of glioblastoma multiformae. No patient demographics (name, age, anatomic site, date of death) were released in compliance with HIPPA confidentiality covenants. The neuropathological criteria for the diagnosis of glioblastoma multiformae followed the World Health Association definitions. Hematoxylin and eosin staining in histology and supporting immunohistochemistry studies of tumor with GFAP protein staining confirm a Diagnosis of GBM.

Fluorescence in Situ Hybridization for detection of Borrelia genes

All coded and anonymous glass slides were thoroughly de-waxed by immersion of the slides in triplicate serial 100% xylene solutions, and then immersed in graded descending solutions of isopropyl alcohols of descending strength from 100% to 70% in water terminating in immersion in tap water alone. The slides were individually processed in sequential single DNA probe FISH hybridization with four DNA probes; two probes were specific for burgdorferi borrelia gene targets and two probes specific for borrelia miyamotoi gene targets.

Five microliters of a single Borrelia DNA probe in a working dilution in a hybridization buffer solution of TE buffer ten mmol with 3.5 mmol MgCl2 pH 8.0 were spotted on the glass slide mounted tumor tissue slices. One thousand microliters of 100% dimethylformamide (DMF) were then flooded over the DNA probe in hybridization buffer on each slide. High stringency FISH was conducted at a constant temperature of 72 degrees C. for 30 minutes under a flexible plastic coverslip to prevent drying of the hybridization mixture. Heating was then discontinued. Slides were allowed to return to 24 degrees C. Each slide was individually hand washed three times in sterile phosphate buffered saline pH 7.4. Slides were air dried and glass coverslips were applied over nonfluorescent 30% glycerol in distilled H20.

DAPI fluorochrome staining of human DNa in tumor cell nuclei

Unstained tumor tissue sections received two drops of a special coverslip mountant solution containing DAPI stain for imaging of human DNa contained in the cell nucleus. A glass coverslip was placed over the liquid coverslip DAPI stain fluid and the slides were examined microscopically after 30 minutes to permit the DAPI fluorochrome to bind to human DNA. Two DAPI coverslip antifade reagents produced identical results in paired examinations; with DAPI was confirmed with , Prolong Diamond Mountant, Themo Fischer Invitrogen Inc, Catalogue P36962 and separately confirmed with Drop N Stain Everbrite Mounting Medium with DAPI, Biotium Inc. Catalogue 23009-T.

Hematoxylin and eosin stain mapping of tumor cell nuclear human DNA content

Standard histology was completed with hematoxylin and eosin staining of the biopsy material to visualize the topographical locations of viable GBM tumor versus necrotic GBM tumor, and to analyze the characteristic dense sheet-like growth of GBM as well as associated GBM neoplastic capillary proliferations inside the glial malignant cell zones. Routine histology was completed with Hematoxylin , (Mayer's Hematoxylin, Cancer Diagnostic Inc CM3953) and with Eosin, (Eosin Y, modified alcoholic, TYR Scientific TS7 500) according to the manufacturers" recommendations

Amyloid stained biofilms and calcofluor carbohydrate stain of biofilms

Congo Red; (American Mastertech Inc). and Calcofluor: Calcofluor white (Neogen Megazyme Ireland Product C-CLFR-5g) stains were completed according to the manufacturers' recommendations. Positive and negative controls produced expected results in reciprocally stained tissues. Control slides containing biofilms of borrelia confirmed Congo Red and to Calcofluor white staining of biofilm communities.

FISH DNA probe Reagents for borrelia species gene target sites

Borrelia burgdorferi B31 strain DNA probe for **gene bbo 0147 for the flagellin B gene of *borrelia burgdorferi:***

5'- (FITC)-cacggt-**TAATCTTACCAGAAACTCCC**-accgtg –(Dabcyl)-3' {green color)

DNA probe for gene **bb00740 inner cell membrane** [common to thirty *borrelia burgdorferi* strains (GenBank) sequences are as follows:

[5" -(FITC) -cgcgag**ATATATTCAAGCAAATTCGATGA CATC**-ctcgcg-(Dabcyl -)3']. (green color)

Borrelia Miyamotoi strain LB2001 DNA probe for **gene Miyamotoi Flagellin B** (BA1_FlaB_MIY_mb3)

5' (-Cy5) -cgtccg-**CGTCAGCCATAAATGCTTCCAGAAATAA-**ccgagc –(BBQ650)-3' (red color)

Borrelia miyamotoi Strain LB2001: DNA probe for **gene GlpQ of *borrelia miyamotoi*** ,(BA2_glpQ_MIY_MB3) (yellow color)

5' – (Cy3)-cgtcgg-**AGAACATACCTTAGAAGCTAAAGCATATGC**-ccgagc-(BHQ_2)-3' (yellow color)

A Nonsense DNa probe Gn2, which by design functioned as a negative control in FISH studies was designed such that its structure does not correspond to any known DNa sequence in GenBank deposits at the NCBI NIH site.

5' (-Marina Blue NHS AmMC6)-cgcgat-**TCTCCGAACGTGTCACGC**-gtcgcg-(BHQ_1)-3' {Nonsense probe]

Monoclonal antibodies for immunohistochemistry of borrelia protein OSP A deposits

Antibody to protein OSP A of borrelia burgdorferi: Murine monoclonal CB 10 (Benach and Coleman 1988) [15] was labeled with a red color fluorochrome using the Biotium Mix-N Stain C660r labeling kit Catalogue 92243. The binding of CB10 antibody to Borrelia burgdorferi protein OSP A was detected as bright red color fluorescence signals on the surface of intact borrelia spirochetes inside tumor tissue. Additionally, cell free OspA protein deposits in individual GBM tumor cells were seen. Cell free biofilm polymer matrix sites in biofilm communities inside tumor tissue also bound the OspA monoclonal antibody.

A Conjugate of Staphylococcal protein A with Horseradish peroxidase ,(GenScript HRP-Protein A, Catalog M00089) was deployed in IHC to identify sites of monoclonal Ab binding to borrelia protein OspA deposits associated with GBM tumor cells. Diaminobenzidine (DAB) chromogen) was deployed according to the manufacturer's recommendations (Vector Laboratories Impact DAB Substrate kit, Peroxidase, SK-4105.) Tissue bound Staph protein A conjugated to HRP produces insoluble deposits of DAB brown chromogen at the sites of MAB CB10 antibody binding in IHC. This reagent enhances the definition of borrelia bacterial protein OspA deposits inside tumor cell cytoplasmic sites and inside of tumor cell nucleus sites. OspA protein deposits biofilm matrix sites are also well defined with DAB color deposits.

Microbe controls for FISH and for IHC

American Type Culture Collection ***borrelia burgdorferi*** strain B31 (ATCC 35210) was grown in vitro to log phase and thin smears on glass slides were heat fixed and postfixed in 91% isopropyl Alcohol and served as a control for the OspA monoclonal IHC studies. Catherine Brissette, PhD generously provided thin smears on glass slides from a pure culture *Borrelia miyamotoi* strain LB2001. And these served as both positive controls for DNa FISH hybridizations and served as negative protein controls for IHC with MaB CB10 In IHC studies.

BLASTn GenBank documentation of specificity for DNA probes. (Data not shown here)

BLASTn supercomputer interrogations of the nucleotide sequences of each DNA probe confirmed 100% matches with DNA in all of the probes utilized here. GenBank data sets of complete DNA sequencing off *borrelia burgdorferi* and *borrelia miyamotoi* deposited strains showed no mismatches and no gaps with intended reference NCBI GenBank Borrelia species targets. The Negative control Nonsense reagent GN2 in BLASTn interrogation returned no matches with any life form in the USA GenBank, NCBI, NIH registry of all known life forms.

Fluorescence microscopy methods

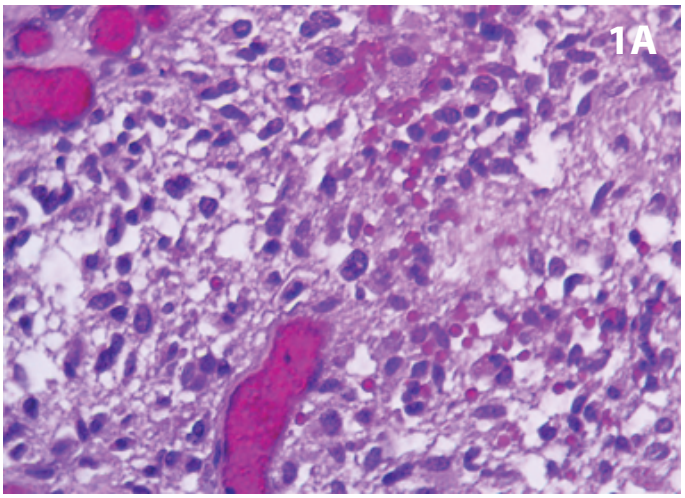
DNA probe FISH hybridizations of patient GBM tissues and bacterial control slides were illuminated with bright monochromatic from light emitting diodes (LED) (Red, Yellow, Green, ultraviolet) appropriate for the excitation of fluorochromes Cy5, Cy3,FITC attached to the DNA Probes. Calcofluor white staining produced fluorescence under 390 nm illumination. The nonsense reagent Molecular Beacon DNA probe was non- reactive in FISH studies. Photomicrographs were captured through a trinocular mounted 16-megapixel digital camera.

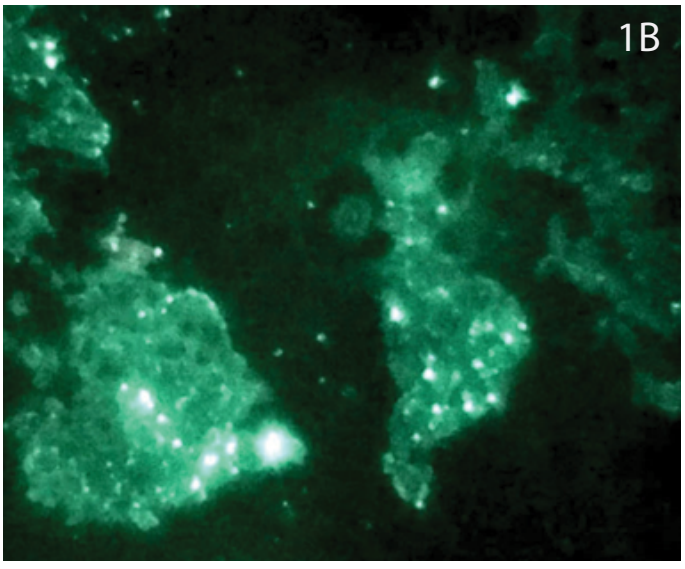
Monoclonal antibody CB10 fluorescence Immunohistochemistry specific for detection of *Borrelia burgdorferi* outer surface protein A (OSPA) was completed [15]. A red color fluorescent label, Biotium Mix-N stain CF660R fluorescent chromogen was deployed in a working dilution of the conjugate (1/80 of stock in RPMI tissue culture medium) which was flooded over unstained tissue sections and incubated at 24 deg C for 30 minutes. The slides were then washed three times in PBS Ph 7.4 and examined in fluorescent microscopy. After completion of fluorescence microscopy with the CB10-Biotium CF66OR red fluorochrome, a second IHC label was layered over the same slides carrying a Horseradish peroxidase label and brown color DAB reaction product at the site of MAB CB10 tissue binding. Sites of CB10 MAB binding in tumor tissue were reconfirmed by DAB staining .The topography of MAB binding sites in the cytoplasm versus the nucleus compartment of the GBM tumor cells was reconfirmed with this non-fluorescent technology.

Results

Human DNA topography in DAPI stains and topography in Hematoxylin and Eosin stains: (Figures 1 A,B,C).

Hematoxylin and eosin stains (Figure 1A) confirmed the expected histomorphology of glioblastoma multiformae in all patient specimens. Brain fragments with normal low cellularity and benign histology (devoid of GBM)were microscopically





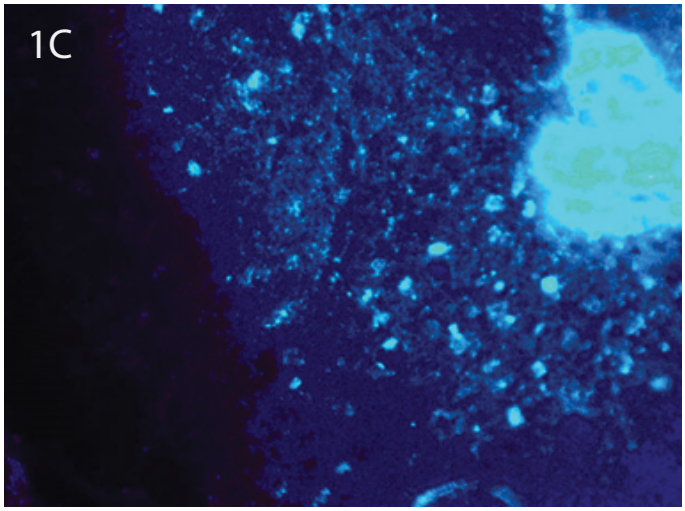


Figure 1. **A:** *Glioblastoma multiformae tumor - Hematoxylin and Eosin stain Human DNa stains dark blue with Hematoxylin 400x original magnification;* ***B:*** *Glioblastoma multiformae tumor – Fluorescence in situ hybridization (FISH) Borrelia bacterial DNA stains Green (fluorescent stain) 400x original magnification;* ***C:*** *Glioblastoma multiformae tumor – DAPI stain for human DNA in nuclear sites of tumor cells, Human DNA stains turquoise inside of tumor cell nuclei, 400 x original magnification*

absent in all patient biopsy material. (Data Not Shown here). The dimensions of individual malignant GBM tumor cells in hematoxylin and eosin-stained brain tumor biopsies ranged between eight to twelve microns in diameter. High-density tumor cells in sheets were demonstrated as arrays of closely packed tumor cells. Tumor cell to tumor cell boundaries between cells varied in linear spacing in various microscopic fields of view and zones of overlap between serial focal planes of optical resolution were encountered as expected. Histological stigmata feature of GBM seen in five patients were manifest in the following categories.: Bizarre hyperchromatic tumor cell nuclei, tumor cell hypercellularity, frequent mitotic figures, cellular necrosis of tumor, palisading tumor cell arrays, and bizarre capillary type proliferations.

DAPI stains bound to human DNa which was contained in the nuclei of malignant glial cells assessed the "cell density of Human DNA in neoplastic cells." (The spaces between malignant glial cells did not show DAPI blue color fluorescence). (Figure 1C). Fluorescent In situ Hybridization [FISH] studies of Borrelia bacterial DNA deposits: (Figure 1B) (Figure 2A) assessed the "bacterial infection cell density".

Bright signal green color, red color, and yellow color fluorescence of DNA probes bound to "bacterial DNa" inside the nuclei of GBM tumor cells reported the relative magnitude of bacterial infection . Also identified were Borrelia DNa deposits inside biofilm communities. Green color fluorescence signals documented *burgdorferi borrelia* DNA and red color(Figure 2B) and yellow color fluorescence signals (Figure 2C) documented *miyamotoi borrelia* DNA sites. Side by side tissue comparisons of DNA probe hybridizations with green color DNA probes and with separate red color and yellow color DNa probes documented simultaneous double species (*burgdorferi* and *simultaneous miyamotoi*) infections in GBM tumor sites.

Whole *borrelia burgdorferi* spirochetes infiltrating the interstitial regions (Figure 3A) between individual glioblastoma tumor tissues were identified in five of five GBM patient

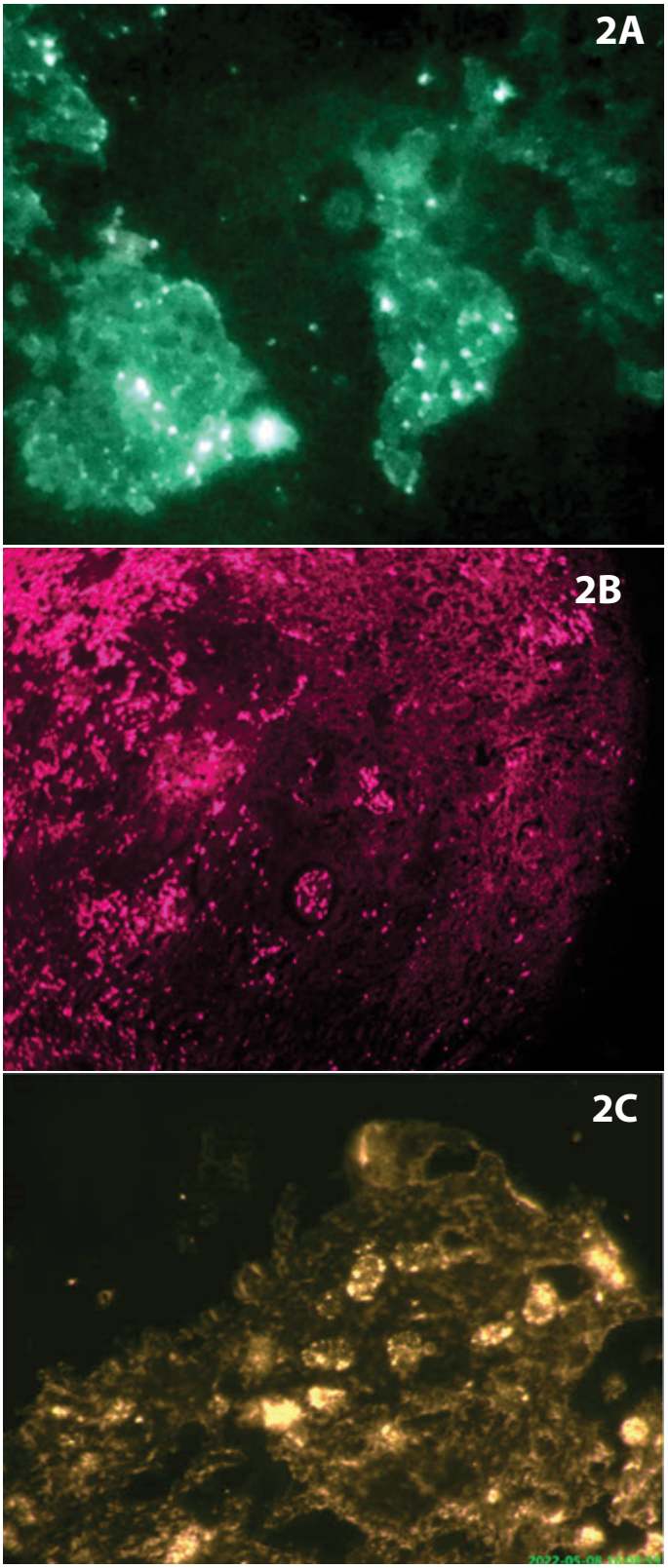


Figure 2. *A: Glioblastoma multiformae tumor- FISH with borrelia burgdorferi DNa probes bbo 0147 and Bbo 0740 (green color signals indicate bacterial DNa inside sites of Tumor) 400 x original magnification; B: Glioblastoma multiformae tumor- FISH with borrelia miyamotoi DNa probe for Miyamotoi flagellin gene Miya Fla (red color signals indicate bacterial DNa inside sites of Tumor), 400x original magnification. C: Glioblastoma multiformae tumor – FISH with borrelia miyamotoi gene for GLPQ (yellow color signals indicate bacterial DNa inside sites of tumor), 400x original magnification.*

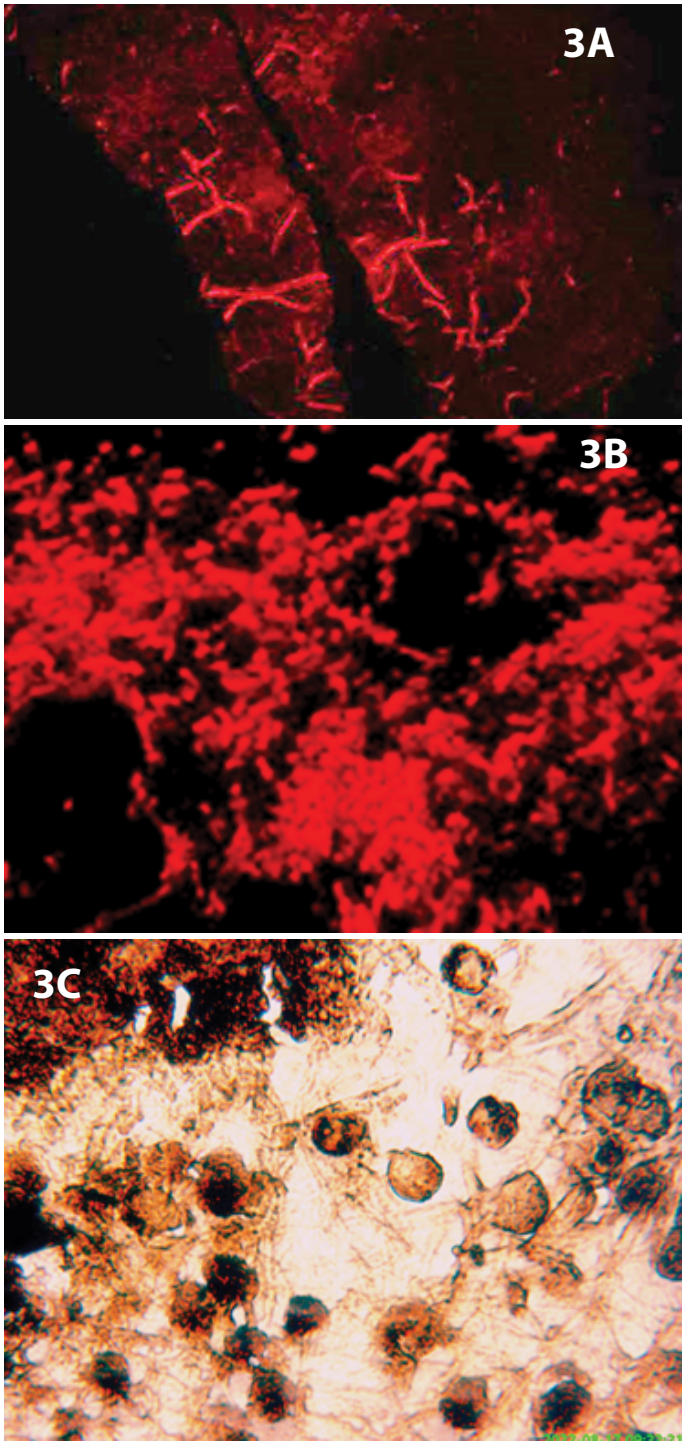


Figure 3 *A: Individual borrelia burgdorferi spirochetes inside tumor tissue biopsies (Red color signals indication IHC binding of borrelia protein with Monoclonal antibody CB10 to borrelia burgdorferi major outer surface protein A [OSP A], 400x original magnification; 3B : Total tumor cell OSP A deposits inside tumor tissue biopsies (Red color signals indicate IHC binding of Monoclonal antibody CB10 to borrelia burgdorferi major outer surface protein A [OSP A], 400x original magnification. C: IHC study with Monoclonal Antibody CB 10 bound to protein OspA inside the cytoplasm and inside the nucleus of individual tumor cells (black color indicates deposits of Chromogen Diaminobenzidine (DAB) 400x original magnification.*

biopsies. Simultaneous whole *borrelia miyamotoi* spirochetes in FISH hybridization studies were seen in all patient biopsies.

FISH fluorescence signals covered the non-necrotic GBM tumor biopsy particles abutted against necrotic tumor zones and zones of clotted blood adjacent to viable tumor in many microscopic fields of view. (Figure 3B) Fragmentary remnants of whole spirochetes with all DNA probes were contained in interstitial spaces between GBM tumor cells. Fragments of broken borrelia cylinders in both cytoplasmic and nucleus compartments of tumor cells were imaged. (Figure 3C)

Observation of microbial DNA deposits inside individual tumor cell nuclei of GBM tumor cells (Figure 2C -yellow color punctate fluorescence signals in nucleus sites) is a first of kind discovery and these data argue in favor of a process whereby the colocalization of microbial DNA with human DNa might argue for a mechanism for local intra-tumoral and intertumoral bacteria deposits as a stimulus or as a promoter for neoplastic uncontrolled cell division of glial cells.

Capillary blood vessels between groups of GBM tumor cells produced bright signals in FISH with *borrelia burgdorferi* DNA probes and with DNA probes for *Borrelia miyamotoi*. Both species of borrelia have been documented to invade the endothelial cells of blood vessels in benign biological settings and in in vitro laboratory studies. (Data not shown)

Immunohistochemistry for detection of *borrelia burgdorferi* protein OSPA with monoclonal antibody CB 10 [15] produced strong red fluorescent signal detection of borrelia Specific protein antigen OspA on surfaces of GBM tumor cells. Bright field white light illumination of DAB-stained tumor cells after IHC with Staph protein A- Horseradish /DAB chromogen development precisely defined the intersections of spirochetes and proteins of spirochetes inside malignant brain sites. The optical imaging of intracellular whole borrelia spirochetes attached the surface of individual tumor cells and resident inside GBM tumor cells in the cytoplasmic and in the nucleus, compartments provide additional support for infection and neoplastic cell growth. Sites of DAB positive IHC bright signal reactivity (Black/brown color) inside GBM tumor cells provided superior optical resolution over fluorescence techniques to distinguish separate cytoplasmic space infections from nucleus compartment infections.

Biofilms of live borrelia associated with Tumor: (Figure 4A,4B,4C,4D,4E)

Biofilm communities of borrelia spirochetes percolated throughout the GBM tumor tissue biopsies. The DNA of *borrelia burgdorferi* (green color products) (Figure 4C)and DNA of *borrelia miyamotoi* (red color products) (Figure 4E)hybridized to cell free DNA deposits in the biofilm matrix. FISH method hybridizations also disclosed live borrelia inside the biofilm matrix sites.(Figure 4C) DAB stains again provided superior optical imaging of Individual spirochetes, and fragments of spirochetes inside biofilm communities. Discrete borrelia biofilms inside GBM tumor sites were also visualized as Congo Red Amyloid stain as positive oval shaped plaques attached to GBM tumor tissue. (Figure 4A) Discrete borrelia biofilms inside of GBM tumor were also imaged in blue color fluorescent Calcofluor staining for carbohydrates in the biofilm matrix. (Figure 4B) Specialized granular form “dot-like” live borrelia were present inside polymeric matrix communities where cell free matrix polymer volumes exceed volumes of matrix containing live borrelia.(Figure 4D) The granular morphology

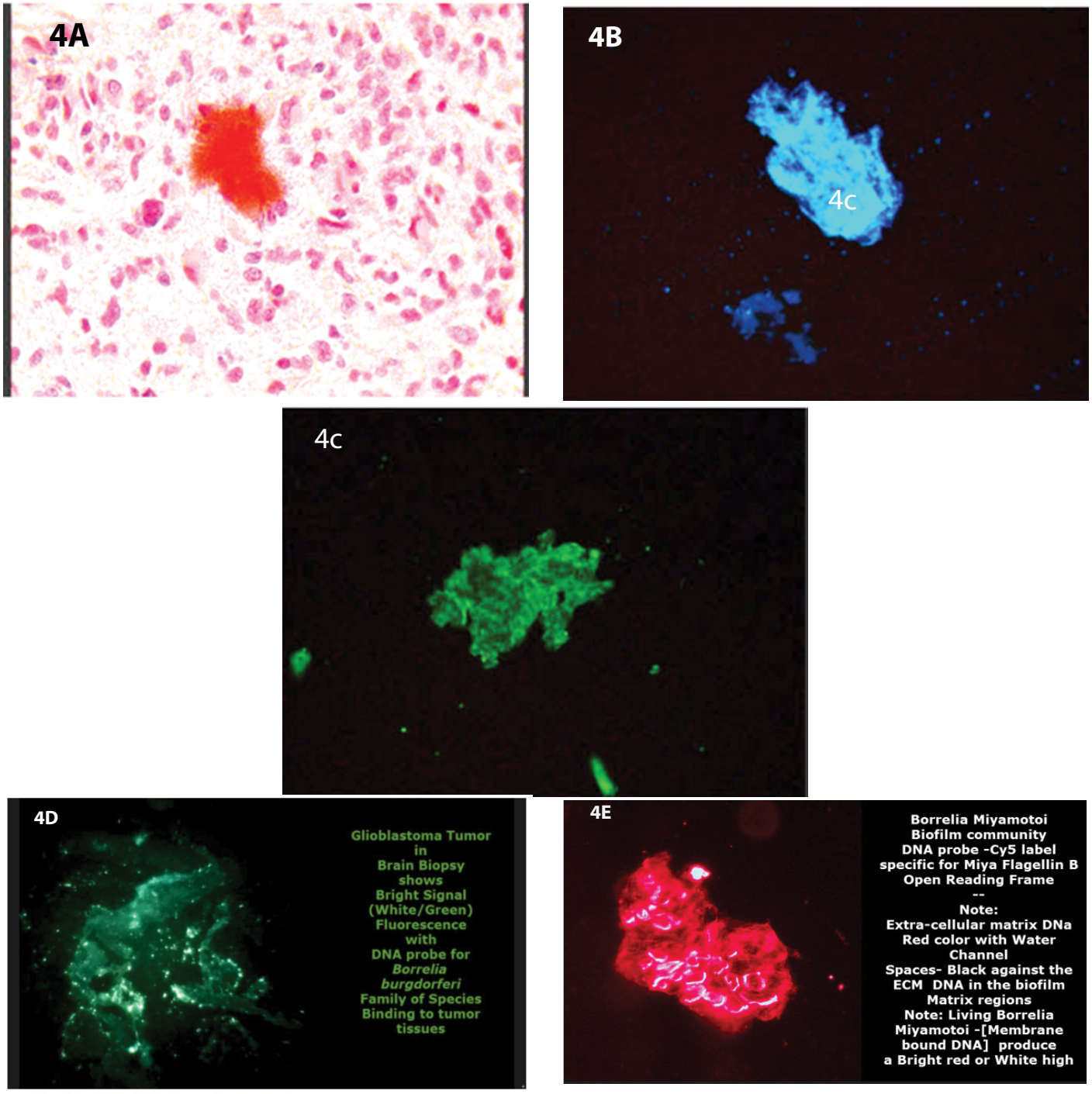


Figure 4. *A: Biofilm of borrelia deposit with abundant cell free polymer matrix which binds human amyloid proteins in Congo Red stain in a borrelia biofilm community, 400x original magnification; B: Biofilm of borrelia which bound Calcofluor white stain for biofilm carbohydrate deposits in the polymer matrix [Blue color fluorescence] , 400x original magnification; C: Biofilm of borrelia deposits of cell free DNA inside the matrix of the biofilm with FISH hybridization using DNA probes bb0 0740, 400x original magnification. D: Whole biofilm of borrelia burgdorferi detached from underlying tumor tissue demonstrating abundant green color Borrelia DNa fluorescence hybridization signals in the polymer matrix sites and brighter green signals from live borrelia inside the biofilm, 400x original magnification; E: Whole biofilm of borrelia miyamotoi detached from underlying tumor tissue demonstrating abundant red color fluorescence hybridization fluorescence signals of cell free bacterial DNA from cell free polymer matrix sites and brighter white/ red signals from live borrelia miyamotoi inside the biofilm community. 400x original magnification.*

live borrelia units outnumbered the spirochetal cylinder-shaped units inside the majority of biofilm communities. Biofilms were abundantly present inside multiple levels of serial histological sectioning of tumor biopsies. Amyloid reactive biofilms and Calcofluor reactive biofilms were imaged in diverse planes of optical focus above and below the focal planes of sheets of tumor cells and amyloid related stains assisted in defining existence of permeative borrelia biofilms in multiple optical planes of resolution.

Conclusion

FISH hybridization study of five human decedents with glioblastoma multiformae using multiple borrelia species specific gene target DNA probes demonstrated that dual species borrelia infections (*borrelia burgdorferi* infection and *borrelia miyamotoi*) were present in infected human glial malignant tissues in all five patients. Single borrelia spirochetes representing *burgdorferi* group and *miyamotoi* groups of borrelia species, and their biofilms were separately confirmed with FISH specific DNA probes.

Multiple previous peer reviewed reports have established that borrelia spirochetes of the *burgdorferi* family of pathogens and of the relapsing fever borrelia group of pathogens avidly associate with and bind to benign human glial cells in brain sites and to tissue cultured glioma harvested tumor cells in in vitro tissue culture models [1-14]. Beyond mere cell surface attachment of borrelia to live glial cells in laboratory in vitro studies, herein is abundant evidence in vivo that borrelia spirochetes penetrate the tumor cells in GBM. Overt nucleus compartment glial cell invasions inside of glioblastoma multiformae tumor cells suggests a novel pathogenesis pathway of possible bacterial DNA corruption of human DNA when co-localized inside the human glial cell nucleus. This is the very first report of evidence of such co-localization by virtue of both bacterial protein signatures and by bacterial DNa signatures of two species of *borrelia (burgdorferi and miyamotoi)* inside a human glial cell malignancy. Total DNA inside the malignant glial cell nuclei of five GBM patients was represented by the sum of DNA species from three separate living organisms: Human glial nucleus DNa, Bacterial agent *borrelia burgdorferi* and bacterial agent *borrelia miyamotoi*. It is sobering to contemplate how any human cell, so parasitized, might possibly ever maintain a normal biology.

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The author appreciates the special unique opportunity to review five anonymous decedent human glioblastoma brain biopsies provided by neuropathologists from the Long Island New York metropolitan region.

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Dr. Ali Javid PhD designed the Dna probes, synthesized, purified, and validated the probes in Melting curve analysis at Gene Link Inc. Hawthorne, N.Y.

Positive control glass slides with pure cultures of borrelia miyamotoi strain LB2001, were gifted to the author by Catherine Brissette PhD.

Conflict of interest declaration

The author declares that he has no conflicts of interest and has no commercial interest in connection with any aspect of this research.

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Supplementary Data

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