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Biomarkers for early detection of high risk cancers: From gliomas to nasopharyngeal carcinoma

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Abstract:
Nasopharyngeal carcinoma (NpC) is a malignant disease associated with Epstein-Barr virus infection, and often diagnosed at an advanced stage. This significantly curtails patient survival. We hypothesize that a panel of biomarkers can be assembled to assess NpC incidence, early detection, and tumor progression during therapeutic intervention. Our thesis rests on a model of successfully predicting high-risk gliomas by means of a carefully crafted panel of molecular mitotic biomarkers (i.e., securin, survivin and MCM2). The strategy we propose holds strong promise for prevention and cure of NpC. The approach we propose seeks to identify certain biomarkers from viral materials, patient tissues and assessment of related diseases, whose signatures, taken together, will be endowed with some degree of congruency, or sense of a coordinated language (i.e., "votes"). Biomarker "voting" will then permit to outline a broad coordinated molecular map for the molecular and epigenetic characterization of each individual patient's NpC tumor. We will draw on the process of contrasting biomarkers in health and disease, which rests on the auto-proteomic concept particularly relevant in high-risk cancer individuals, such as is the case for NpC. In brief we defend, current advances in human proteome profiling proffers the possibility of having individual baseline proteomic profiles using local body fluids (e.g., saliva, nasal secretions, sputum) or systemic fluids (e.g., plasma, serum, cerebrospinal fluid) to unravel a personalized molecular map for high-risk NpC individuals. Regular check-up will monitor for new or impending manifestations of NpC, and provide a secure assessment of incidence and early detection.

Keywords: Molecular & Epigenetic Biomarkers, Nasopharyngeal carcinoma, Translational evidence-based personalized medicine, Proteomics, Auto-proteomics, Epstein Barr virus, Gliomas, MCM2, Survivin, Securin, Protein voting, serial analysis of gene expression (SAGE)

Background:
Nasopharyngeal carcinoma (NpC) is a malignant tumor mostly of the lateral wall of the nasopharnyx. NpC has multifactorial etiological factors, including genetic, environmental, and Epstein-Barr (EBV; human herpesvirus-4, HHV-4) infections **[1, 2]**. NpC has a significant geographical distribution **[3]**, being most common among Asians, particularly the adult population of Southeastern Asia (prevalence: 15-50/100,000, 37) **[1, 2]** and North African (prevalence: 15-50/100,000, 37) **[1, 2]**. In Africa, NpC is the most frequent childhood cancer. Whereas prevalence of NpC is generally 2:1 higher in males, compared to females **[1, 37]**, increased risk for NpC is associated with specific class I human leukocyte antigens (HLA-1) haplotypes. across genders (12,13): HLA-2, HLA-B17, and HLA-Bw26 haplotypes double the risk of NpC, as well as epigenetic aberrations in chromosomes 1, 3 , 9, 11, 12, and 14 **[37].**

Diet plays an important role in individuals at high-risk for NpC. Regular consumption of salted fish, particularly from an early age onward **[5, 12, 15, 17, 18]**, and consumption of preserved foods **[19]** increase incidence. Exposure to cigarette smoking and other sources of nitrosamine **[5]** as well as to polycyclic hydrocarbons also increase risk for NpC. Chronic nasal infections, poor oro-nasal hygiene, inadequate nasopharynx ventilation, and improper use of certain herbal teas, concoctions and medicines, such as intranasal oils, have all been reported to predispose NpC development **[5, 14, 16].**

The nasopharynx is composed of stratified squamous cells, pseudostratified columnar ciliated cells, and patches of some intermediate cells (1). Histopathologically, NpC takes one of three patterns: (1) keratinizing squamous cell carcinomas (type I), (2) non keratinizing squamous cell carcinoma (type II), or (3) undifferentiated carcinomas (type III) with abundant non-neoplastic lymphocytic infiltrate **[1-3]** of putative tumor-directed cytotoxic CD8+CD38+ tumor-infiltrating lymphocytes (i.e., TIL).

A DNA Herpes virus that targets B lymphocytes by binding its viral envelope glycoprotein (gp350) to the complement component-3d receptor 2 (CR2, CD21), and its gp42 to the leukocyte antigen, HLA **[5],** EBV infects and persists in the latent form preferentially in B cells **[10].** The virus, first isolated in Uganda in 1964, is associated with several cancers besides NpC, including gastric carcinoma, B cell lymphomas (e.g., Hodgkin's and Burkitt's lymphomas) **[5, 29],** and a subset of T cell lymphoma **[20].** EBV is also implicated in infectious mononucleosis **[21].**

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The EBV genome occurs in the epithelial cells of most type II and III carcinomas **[3, 4],** and its latent membrane protein (LMP)-1, the main viral transforming protein, has pleitrophic effects in eukaryotic cells that range from increase signaling through the TNF-α/CD40 pathway, induction of anti-apoptotic proteins, increase cell surface adhesions, and expression of certain cytokine genes **[5-9].** LMP2A, but not LMP2B can (1) favor survival and proliferation of B cells in the absence of signaling through the B cell receptor, (2) transform epithelial cells and enhance their adhesion and motility in part through the PI3K pathway **[5, 11],** and (3) increase genes involved in cell cycle induction, inhibition of apoptosis and suppression of cell mediated immunity **[5]**.

We propose the hypothesis that the occurrence and progression of NpC can be predicted by assessing a panel of molecular and epigenetic biomarkers. A corollary hypothesis states that a proteome profiling such as that proposed in Schemes 1A-1C (see supplementary material) of local (e.g., saliva) or systemic body fluids (e.g., plasma) may yield a personalized molecular map for high-risk NpC individuals, and that regular check-up will permit monitoring for new or impending manifestations of NpC.

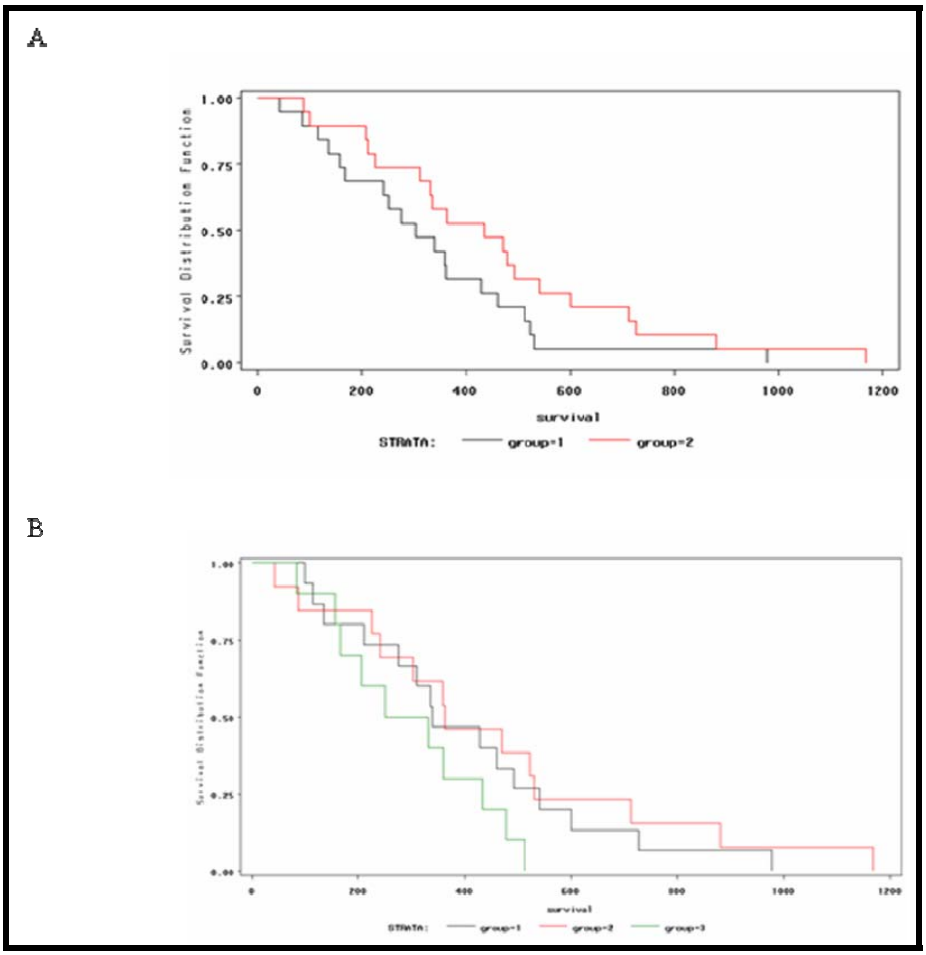

Figure 1: Panel A: Survival graph of glioma patients (control-8 and selected-30) reference by age (55 years): This figure show that group 1 patient (≥55 years) had worse survival compared to group 2 patient (< 55years). The age 55years was used as a reference based on evidences in the literature and the median age estimate of patients used in this study. Survival graph was generated by input of the survival data in the SAS statistical software (X^2, p=0.1241). **Panel B**: **Survival graph of the 38 high risk glioma patients based on positivity for number of markers**: Thirty (30) selected and eight (8) control patients were assessed based on the positivity for the four mitotic markers. The control patients were determined to be high risks group 2a prognostic members (see reference 22) by gene microarray studies. This figure shows that patients that were high risk and positive for two markers (Group 1) or three markers (Group 2) have similar survival pattern and tend to live longer than those patients that are high risks and positive for the four markers (Group 3). Survival distribution graph was generated by input of the survival data in the SAS statistical software (X^2, p=0.4088).

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Methodology:
A panel of molecular and epigenetic biomarkers can be designed, generated and tested with the specific intent of assessing NpC incidence, early detection, and tumor progression during therapeutic intervention. The rationale for this methodology rests on current understanding that regulation of genes, including oncogenes and tumor suppressor genes, is associated with heritable traits encoded directly in the genome.

Heritable non-encoded aspects of the genome can control and modify gene expression. Such epigenetic modifications are passed down the cell progeny, though not directly encoded in the genomic structure. They, nonetheless, alter the physical availability of genes to transcriptional machinery. Epigenetic modifications, including DNA methylation and histone acetylation, can therefore exert modulatory control over the expression of certain oncogenes and tumor suppressor genes. This is typically obtained by modification of the chromatin architecture, assembly and repair events, which result in open "euchromatin" or closed "heterochromatin" states **[38]**.

A model of successfully predicting high-risk gliomas by means of a carefully crafted panel of molecular mitotic biomarkers (i.e., securin, survivin, topoisomerase-II alpha [TOP2A], and minichromosome maintenance-2 [MCM2]), and the resulting protein votes of molecular mitotic signatures (Figure 1a & 1b) demonstrates that high expressions of at least two of these markers were pooled together as protein votes to identify very high-risk glioma patients belonging to Group 2a prognostic group **[22]**. The panel of biomarkers, which consist of securin, survivin an anti-apoptotic protein and MCM2, was found to be reliably effective in the identification of high-risk patients with molecular signatures similar to that of control patients, who were established to have the known high-risk Group 2a prognosis, based on the traditional gene microarray analysis (Figure 1a & 1b).

In the case of NpC, we propose a methodology directed at the assessment of a panel of biomarkers designed to predict patients at high-risk for NpC. We project that the panel will involve, in addition to the traditional patho-anatomical assessment of masses and enlarged drainage lymph nodes, certain molecular and epigenetic markers consequential to EBV infection (e.g., anti EBV antibodies, viral capsid antigen, EBV nuclear antigens [EBNA-1 & 2], latent membrane proteins 1 & 2, EBV RNA-EBER-1 & 2 [EBV non-polyadenylated RNA's], and microRNA's), as well as lymphocytic markers (e.g., TIL, flow cytometric measures of CD23, CD30, CD39, CD58 & CD70, and ELISA of IL-10, all of which have been reported to rise in clinical and experimental models of EBV infection; 5,23,24), as well as DNA, RNA and chromatin signatures by a variety of protocols including serial analysis of gene expression (SAGE) (33). Expectations are that marker identification would be successfully carried out in body fluids local to the NpC tumors (i.e., whole, parotid sub-mandibular/sub-lingual saliva, sputum and expectorate, nasal discharge), as well as systemic fluids (i.e., serum, plasma, cerebrospinal fluids, thoracic lymph). Histological and histopathological control verification would derive from assessment of normal tissues for signs of NpC risk, swab of oral mucosa, oro-pharynx, nasal and nasopharynx by means of DNA analysis, including culture/serological analysis of viral materials. The auto-proteomic protocol for EBV titers in body fluids, and immunohistochemical analyses for markers of mitosis (e.g., securin, survivin and MCM2 as in our glioma model), will then need to be supplemented by assessing viral particles and phospho-proteins (e.g., PI3K pathway, altered by ELMP2A, as noted above) **[5, 11]** in order to assess NpC occurrence and regression during treatment.

Discussion:
The recent advances in proteomics and its utilization to analyze blood, plasma, saliva and related body fluids are vital to the studies of cancer particularly in the search for biomarkers. These tools can now be harnessed to pre-empt the occurrence of cancer, particularly in high-risk patients with NpC, because this particular tumor is most often diagnosed after the disease has advanced to its later stages. When a mass in the neck first becomes evident, the cellular events of the cancer are substantial, and the tumor is likely to be metastasizing. Thus, patient survival is curtailed. The hypothetical proposition that molecular and epigenetic biomarkers, which we now have the capability to test and assess with reliable stringency in body readily accessible body fluids (e.g., saliva, **[39]**), may be used as likely informants for NpC early detection and monitoring of therapy outcome.

Auto-proteomic approach towards biomarker discovery in NpC
We propose here that NpC and related molecular and epigenetic signs during the transition of normal tissue to NpC must be picked up by proteomic protocols prior to gross tumor development and anatomical pathology, because of the inherently greater strength and sensitivity of molecular over traditional histological techniques. The rationale for our hypothesis rests on the awareness that fundamental physiological and pathological changes that become associated with disease occurrence are antecedent to tumor growth and metastasis. Furthermore, the increased reliability in proteomic mining of the body fluid proteome [25] leads us to infer that an auto-proteomic approach protocol may be essential for individuals at high-risks for NpC (e.g., based on genetic make-up, exposure history, diet).

The auto-proteomic protocol **(Scheme 2 in supplementary material)** should provide a high-risk individual's marker profile during the health period and before the tumor growth commences. This initial "baseline" profile would then be compared at regular intervals, and tested for the emergence of proteomic markers suggesting the onset and presence of NpC. The regular update of the proteomic profile, the auto-proteomic test of a high-risk subject will in this manner ensure personalized evidence-based medicine for NpC patients, prompt intervention, and reliable evaluation of the

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effectiveness of the treatment intervention. It is clear that the results we have obtained with our glioma model point to implications and applications specifically to the NpC case, as discussed here. It is also evident that our hypothesis can be generalized to other cancers beyond NpC as well.

A pragmatic objection to the model we propose queries what tissue ought to be tested and analyzed in subjects at high risk for NpC in order to optimize the panel of informant biomarkers. We have submitted above that nasal sputum and nasal discharges ought to be tested, and we cited epidemiological data that point to African children, South-Asian adults, and males preferentially as particularly high-risk for NpC. In an approach parallel to the analysis of nasal discharge and sputum for the monitoring of lung cancer **[26-28],** we argue that the same body fluids and whole saliva could be among the tissues certainly of heightened value for NpC monitoring. The quantification of EBV materials and anti-EBV antibodies could be used as a normative value to suggest EBV-positivity and increased molecular and epigenetic propensity (i.e., immunohistochemistry, molecular mitotic profile) to developing NpC.

We defend that saliva, nasal biopsy materials (e.g., by nasopharyngoscopy) for EBV, which has been implicated in the formation of Burkitt's lymphoma, a tumor of the jaw common in African children **[29]**, and for the NpC molecular signature should be scheduled bi-annually in high-risk individuals. This preventive protocol would be no different than the common recommendation of frequent colonoscopies annually for patients at high risk for colon cancer **[30, 36]**.

Translational personalized medicine approach for NpC patients

Translational personalized evidence-based medicine should be followed through at the bench, and in the clinic. The ability to successful culture patient tumor samples **[5],** subject them to molecular analysis, run samples on western blot to probe with antibodies, or extract DNA and RNA from these samples for further assessment, including the use of laser micro-dissection to help isolate specific sites on the tumors to test, brings to us that high level of achieving molecular precision in personalized medicine.

It ought to be possible to obtain gene expression profiles and proteomic signatures of a patient sample, normal vs. NpC tumor, in cell cultures subjected to EBV transformational materials or subjected to array of growth factors, as well as in ligands found in body fluids *in vivo*. Validated markers that result from this approach could be profiled for early detection of disease in the local and systemic body fluids. Progress in saliva diagnostics, including the development of nanotechnology, afford point of care benefits **[31]**, and have established that some degree of personalized medicine on the bench is highly informative in the context of translational evidence-based medical care in cancer diagnostics and prognostics.

The hypothetical protocol we propose here, therefore, suggests that a personalized bench auto-proteomic approach should guide to an improved personalized clinical approach, which will strive to integrate the best available evidence, the clinic, and the bench in a true translational evidence-based medical diagnostic and practice paradigm. One limitation of our hypothesis is that the proposition will involve a large scale but unique molecular analysis of each patient tumor samples with NpC in a bid to specifically obtain proteomic and genetic profiles of each samples. Taken together this information will be most valuable in that it will be collected into a single database. Expectations are that the pooling together of detailed molecular analysis-genes, proteins and RNA of a consistently rising number of NpC samples will permit a Baysian analysis aimed at revealing and validating statistically distinct biomarker signatures common to this tumor across ethnicities, genders and ages.

The need for panel of biomarkers

Oncology studies increasingly demonstrate the need to use multi-faceted panels of biomarkers for cancer detection, diagnosis and monitoring **[32, 34].** Based on the rationale that NpC is not different than other tumor, we seek to develop a panel of biomarkers specific for NpC, which could serve at the level of prevention, early detection, high-risk detection, and assessment during therapy. The panel must be crafted at a level of detail that involves and incorporates looking at multiple factors including EBV positivity, epigenetic modulation of gene and chromatin architecture, assembly and repair, proteins and regulation of gene products (including RISC, the RNA inhibitory signaling complex, **[38]**), signaling pathways and related phospho-proteins, as well as the summative and subtractive effects of these biomarkers.

For example, we venture that early detection of NpC might be revealed by a panel of biomarkers that will identify the EBV materials (e.g., latent proteins), PI3K-derived phospho-proteins, and flow cytometric lymphocytic markers of EBV infection noted above. In our glioma model, we noted that tumors could be stratified by their gene profile into four molecular prognostic groups **[22]**, and that these stratifications could serve to construct a panel of biomarkers with strong prognostic validity based gene voting and, by derivation, protein voting (Figure 1a & 1b). The strength of our model was confirmed by a similar approach adopted in the case of blood cancers **[35]** in which "genes votes" were involved by weighting them together to help in the classification of leukemia.

Voting among the panel of biomarkers in NpC

Here, in the case of NpC, we propose that the same concept holds strong promise, and argue that if genes can "vote" to identify specific prognostic groups, then proteins must also cast be able to votes, which will soon apply to NpC proteomic biomarkers. We now propose that, in the same fashion, we soon could have situations of "RNA voting", and eventually genomic and epigenetic/chromatin voting.

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It is very important in NpC and other cancers to interpret results of cancer molecular signatures as a whole before a detailed focus on a single signature. This holistic molecular approach could be described as the total gene votes, chromatin votes, RNA votes or protein votes of these signatures. This approach, which represents the direction of cancer diagnosis for the next decade of this XXI Century, help harness together the impact of events associated with NpC and other tumors. It will provide insight into signaling pathway cross talks, suggest new therapeutic intervention or targets, allow for better ways to prevent tumor resistance to drugs, and provide signatures on ways of tumor recurrence and path of metastasis.

Votes of genes, chromatin architecture, RNA or protein may be considered on the basis of those unique to a group or well defined cluster as in gliomas described above **[22].** But, it can still be considered as "votes" of players across signaling pathways and different functional maps. Since cancer is an active interplay of many players, we are prepared to predict that a rigorous careful analysis of these votes will provide novel approaches to the management of NpC and other cancers in the near years to come.

Conclusion:

It is our expectation that NpC will have reduced occurrence if we develop the ability to identify aberrant changes in the normal anatomy, histology, physiology and fundamental biochemistry of the nasopharynx. This goal can only be attained by the development and characterization of a pre-emptive panel of biomarkers aimed at early detection for such cases that cannot be prevented, thereby ultimately leading to early diagnosis of NpC without obvious clinical signs or metastasis, and permissive of promising cure. Molecular analysis of tissues from nasal, nasopharyngeal, oral, oro-pharyngeal, saliva, blood and nasal sputum and discharges from individuals at high risk for NpC is timely and critical for improved detection and management of this cancer, and it promises to be for many other tumors. Physicians and pathologists are likely to be making better sustainable translational evidence-based clinical decisions if they rely on molecular signature data, which are typically more stringent, reliable and reproducible than other bench evidence. As molecular signatures are increasingly validated, and cancer tissue heterogeneity is streamlined by proteomic and related techniques, we envisage a time in the near future when laser micro-dissection approaches will utilize these molecular signatures for directed, targeted molecular intervention. A proactive evolution of the traditional pathological reports will have personalized molecular maps supplement the habitudinal histopathology description. Together, histopathology, personalized molecular map, and auto-proteome assessment will converge to achieve well informed translational evidence-based therapeutic interventions optimized to ensure each patient's benefit and treatment utility.

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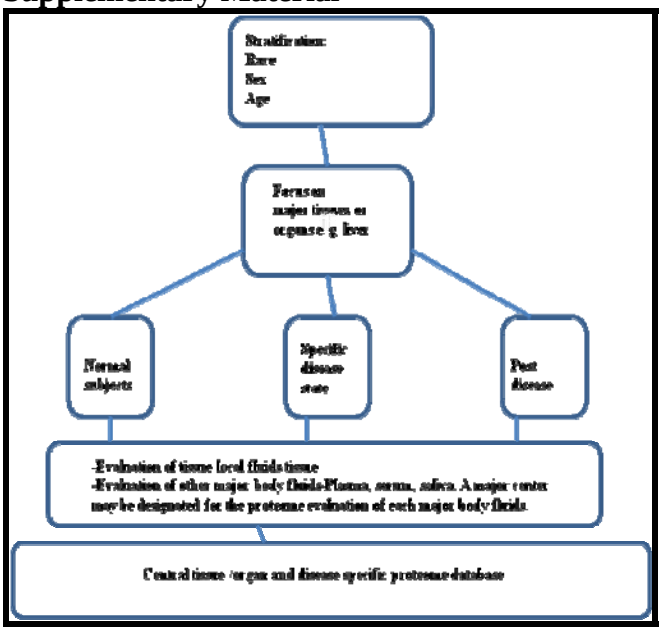
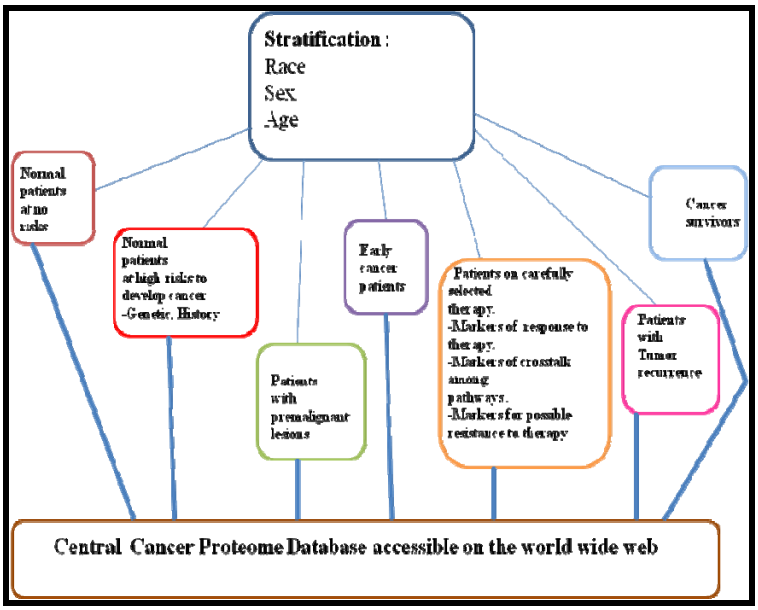
Supplementary Material



Scheme 1A: -Profiling of the human proteome in health and disease. Schematic representation of the process that can be used to generate a human proteome database in health and disease. This approach will offer insight into diagnosis, cure and pre-emptive approach to disease management. While it may appear cumbersome in outlook, major centers for tissue and disease specific proteome project may be established with a database to facilitate cross talk. Depending on the disease, age may be stratified by years as follows: (0-10, 11-20, 21-40, 41-60, >60).



Scheme 1B: Cancer proteome approach. Schematic representation of the approach that could be used to search for valuable biomarkers in NpC cancer patients with a depository into a central cancer proteome database. Suggested stratification by race, sex and age are vital to unravel subtle differences of various cancer presentation, incidence and prevalence. Age may be stratified as follows in years (0-10,11-25,26-30,31-35,36-40,41-45,46-50,50-60,>60). Appropriate modifications may be needed for each type of cancer.

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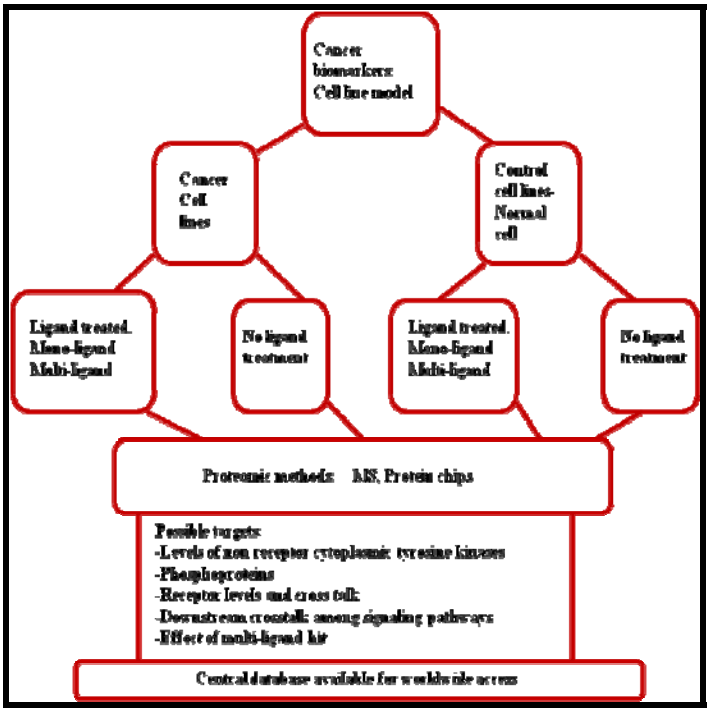
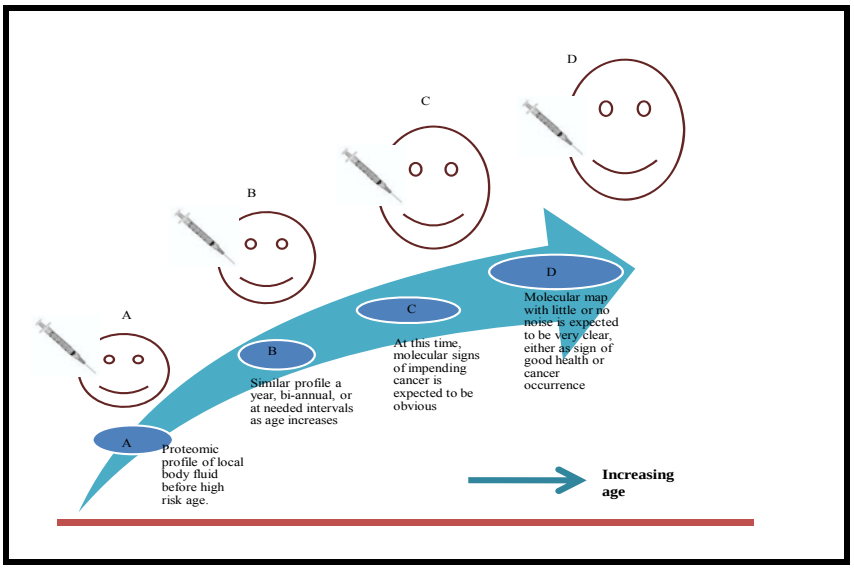


Scheme 1C: Approach to biomarkers-Cell line model. Schematic representation of the process that could serve for the search of biomarkers in cell lines. A designated center for cell line studies could be established to focus on several cancer cell lines. Various cytokines and growth factors (EGF, TGF, PDGF, and FGF) implicated in cancer could be evaluated with a standardized protocol; cells could be hit with multi-ligand to simulate the in vivo dynamics of the body. Possible targets of interests are listed and results obtained from such database could be validated or tested on human cancer tissues/fluids.



Scheme 2: Schematic representation of the auto-proteome approach: Molecular proteomic profile of local body fluids or tissues can be easily assessed in high risks individuals over the period of normal health to the critical ages of development of NpC and beyond. This is assessed regularly annually, bi-annually or at intervals determined by the level of risks in a typical individual. This approach therefore establishes a personal proteomic profile which over time reduces molecular noise and presents with molecular signatures for a normal health or impending cancer. This allows for early tumor detection, increase survival and personalized medicine. This auto-proteomic approach could be use to profile systemic fluids likewise and applied to other cancer model.

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