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Research Article

A Comprehensive Analysis and Prediction of Sub-Cellular Localization of Human Nuclear Receptors

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Abstract. The Nuclear Receptor (NR) superfamily comprises of conserved ligand-modulated intracellular transcription factors which in the presence of their cognate ligands activate a plethora of signaling networks, thereby commencing their respective transcription functions. All NRs are nuclear when liganded or active. However, their localization may differ between nucleus and cytoplasm when unliganded or inactive. NRs control a majority of physiological processes in body ranging from metabolism to reproduction and development. Hitherto, in case of humans, 48 NRs have been identified which are localized either in cytosolic, nuclear or both compartments of the cell. Sub-cellular localization of proteins has great relevance in relation to their function. However, specific sub-cellular localization patterns of human NRs are clouded with ambiguity and are mostly ridden with controversy, with only a few of them being well-studied and established under specific physiological conditions. In the present study, we attempted to bridge the gap and attempted to draw conclusions in relation to sub-cellular localization of human NRs based on published experimental data and by *in-silico* prediction methods. This comprehensive analysis may not only be useful to draw conclusions on their control of physiological processes but may also open new avenues towards understanding of the molecular basis of NR-mediated diseases attributed to their mislocalization and malfunctioning.

Keywords: Nuclear Receptors, sub-cellular localization, *in-silico,* ngLOC, Hum-mPLoc 3.0

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1. Introduction

Nuclear receptors (NRs) are transcription factors which on activation by physiological stimuli, bind to the specific DNA sequences and bring about regulation of complex biological pathways [1]. These receptors function alongside other proteins to regulate the expression of specific target genes, thereby effectively controlling vital cellular functions such as development, homeostasis and metabolism in an organism.

NRs constitute a large superfamily of evolutionarily-conserved proteins. The NR superfamily can be broadly categorized into four subfamilies based on their DNA-binding properties and dimerization preferences. Class I receptors include steroid hormone receptors, such as GR, MR, PR, AR, ER etc., which act as ligand-induced homodimers and bind to the half-sites of target DNA oriented as inverted repeats. Class II consists of receptors which heterodimerize with RXR such as VDR, RAR, TR etc., and bind to the direct repeat half-sites. Class III and class IV receptors are orphan receptors where class III receptors bind to the direct repeat as homodimers while class IV receptors typically bind to extended core sites as monomers [1, 2].

Figure 1: A schematic general structural and functional organization of nuclear receptors.

The modular structure of all human NRs is more or less similar with several independent functional domains. The sequence of N-terminal (A/B) domain (NTD) is least conserved and possesses the activation function-1 region (AF-1). The DNA-binding domain (DBD; constitutes the C domain) which follows the NTD and is highly conserved. It encompasses two zinc finger motifs and is followed by a flexible hinge region (or D domain). Next to the hinge region is a C-terminal ligand-binding domain (LBD; also known as E domain) [3]. Beyond this there may also exist another domain known as the F domain whose function is still ambiguous. Some reports said that this domain has some role in coactivator recruitment to the LBD and thus determines the specificity of LBD and also fine tunes the transcriptional activity of the receptor which were exhibited through the LBD or through the full receptor [1] (Figure 1). As ligand-activated transcription factors, these NRs provide a direct link between signaling molecules that control modulatory processes via transcriptional responses. NRs are promising pharmacological targets as they bind small molecules that can easily be modified by drug design [4]. Thus, these receptors can be utilized to target and cure many major diseases such as diabetes, cancer, osteoporosis, etc. Therefore, it becomes a necessity in itself to possess a prior knowledge of the cellular dynamics and localization of these NRs in their unliganded state so as to be able to target them.

Sub-cellular localization of proteins is crucial for their proper functioning as well as structural organization of all living cells. Mislocalization of proteins has been alluded to the impairment of different cellular processes which ultimately lead to the development of a number of diseases. This further implies that knowledge of localization of proteins in the sub-cellular compartments can help in identifying drug targets in the drug discovery process for various disorders/diseases [5]. When unliganded, NRs may localize either in the cytoplasm, nucleus or both the compartments. However, all NRs are nuclear when liganded, and modulate their target gene transcription. The modulatory activities include either up-regulation or down-regulation of their target genes via multiple mechanisms [1, 6].

There are several reports showing the presence of some NRs like GR, ER and TR in mitochondria, plasma membranes or other organelles where they are suggested to perform non-genomic functions [7, 8]. *In vivo* localization of NRs may be different from the localization pattern observed *in vitro*. It is conceivable that in cultured cells the media conditions can be made ligand-free unlike in case of *in vivo* conditions where the ligand may appear or disappear as per physiological requirements [9]. The generation of a tissue-specific response depends on lineage of a cell in respective tissue [10]. Sub-cellular localization of most of the steroid/nuclear receptors depends on the ligand binding, cell type, physiological state and nature of the interacting proteins [7]. NR-interacting proteins like coactivators and heterodimeric partners can also influence nuclear translocation of NRs [11, 12]. NRs are also regulated by different post-translational modifications (PTM) like phosphorylation, acetylation and sumoylation which may also alter sub-cellular localization. This may have implications in the inception and progression of diseases including different cancers [13].

Generally, the sub-cellular localization prediction tools first take the information about a specific amino acid sequence in a protein and predict the sub-cellular location as the output. The location may be in the cytoplasm, nucleus, endoplasmic reticulum, golgi apparatus, extracellular space, or other organelles. Since, all 48 members of human nuclear receptor superfamily are transcription factors, their significant sub-cellular localization should be either in the nucleus or in the cytoplasm (though a small fraction can be localized in different other cellular organelles). Thus, based on their role as regulators of transcription we primarily focused on these two sub-cellular localizations *i.e.* on cytoplasmic and nuclear localization.

Previously, our laboratory has shown experimentally that human PXR, RXR and ERα are mostly nuclear proteins whereas CAR, AR and GR reside predominantly in the cytoplasmic compartment of the cell [14–16]. However, occasionally reports contradict the established data about subcellular localization of some of several NRs implying that localization of significant number of human nuclear receptors are still in the dark. Therefore, in order to throw some light on the localization of all the 48 members of the human nuclear receptor superfamily we tried to predict their localization through *in-silico* approach.

The ngLOC software can be described as a naïve Bayesian classification method developed to predict the sub-cellular localization of proteins [17, 18]. It is based on utilizing n-grams for computational as well as machine-learned classifications. The ngLOC prediction software holds the capacity to predict a broad range of sub-cellular locations covering as many as 10 sub-cellular locations in the cell. Moreover, customization of this method is possible in order to work with different datasets (both prokaryotes and eukaryotes). In addition to this, the predictions made by ngLOC are exclusively based on the information obtained from the protein sequence. Therefore, this method is apt for making proteome-wide predictions [17, 18]. On the contrary Hum-mPLoc 3.0 (`http://www.csbio.sjtu.edu.cn/bioinf/Hum-mPLoc3/`) is an upgraded version of Hum-mPLoc. It is a powerful tool for predicting human protein sub-cellular localization. It uses an approach where it hybridizes the GO (gene ontology) representation and PseAAC (pseudo amino acid composition) representation. It covers a total of 14 sub-cellular locations. The GO is a controlled vocabulary which is utilized in order to define the biology of any gene product in different organisms [19, 20]. The output obtained is determined using OET-KNN-based predictors (K and λ parameters used). This assembly of classifiers is called Hum-mPLoc 3.0; ‘m’ before ‘PLoc’ implies ‘multiple’, signifying that proteins with both, single and multiple sub-cellular locations can be dealt with using this method; ‘3.0’ indicates that it is an updated version of Hum-mPLoc [20].

It is evident that ngLOC and Hum-mPLoc both can predict more than 10 locations including multiple localizations with ~90% accuracy while other methods are limited by predicting only a small number of sub-cellular organelles with the lack of specificity. In addition, ngLOC and Hum-mPLoc both are more specific and sensitive as these methods are solely based on the smaller protein sequences. Therefore, these methods are highly promising for proteome-wide prediction of sub-cellular localizations. Due to these advantages of these two methods we choose them for predicting the sub-cellular localization of 48 members of human nuclear receptor superfamily.

2. Materials and Methods

2.1. Prediction of sub-cellular localization using ngLOC and Hum-mPLoc 3.0 softwares

The receptor reference sequences were retrieved in FASTA format from UniProt (http://www.uniprot.org) and only these sequences were utilized for prediction. Overall, 48 human nuclear receptor protein sequences were used for prediction of sub-cellular localization. The sequences were then copied into the search box provided at the ngLOC (http://genome.unmc.edu/ngLOC/index.html) and Hum-mPLoc 3.0 (http://www.csbio.sjtu.edu.cn/bioinf/Hum-mPLoc3/) web interface. For ngLOC, the results were obtained in a tabular format with confidence scores (CS) given for the top three predictions along with multi-localized confidence scores (MLCS) (Table I in Supplementary Material available online at http://www.agialpress.com/journals/nurr/2018/101324/). Unlike ngLOC, where confidence scores were also provided along with the final prediction, the predictions obtained in Hum-mPLoc 3.0 provided with only the sub-cellular compartment. The results obtained were tabulated alongside ngLOC for further comparisons (Table I in Supplementary Material available online at http://www.agialpress.com/journals/nurr/2018/101324/). Since the study was focused with nuclear receptor superfamily members, only nuclear and cytoplasmic localization of these transcription factors was considered and projected.

2.2. Cell culture and imaging

In order to give a representative figure of different types of sub-cellular localization in living cells, we transfected 500ng of a GFP-tagged human nuclear receptor in HEK-293T cells and after 24 hours of expression period in steroid-stripped antibiotic free medium, images were captured with a Nikon upright fluorescence microscope model 80i equipped with water immersion objectives and connected to a cooled CCD digital camera (model Evolution VF, Media Cybernetics, USA) and analyzed with Image ProPlus version 5.0 software (Media Cybernetics, USA). The images were processed using standard image processing techniques. For depicting sub-cellular localization, we divided them into five categories depending on intensity of fluorescence observed in the cytoplasm and the nucleus of the cell. If the receptor expression was exclusive to the nucleus and absent in the cytoplasm, we termed it as nuclear (N). If the receptor expressed strongly in the nucleus with marginal fluorescence in the cytoplasm, it was referred to as predominantly nuclear (N>C). When the fluorescence was nearly equal in both nucleus and cytoplasm, it was categorized as N=C. The localization was considered predominantly cytoplasmic (C>N) when the fluorescence was stronger in the cytoplasm as compared to the nucleus. Finally, the receptor was regarded as cytoplasmic (C) when its expression was restricted exclusively to the cytoplasm of the cell (Figure 2). For the present manuscript, for easy understanding we grouped these sub-types of localization into two major categories as nuclear (N + N>C), denoted as N and cytoplasmic (N=C + C>N + C), denoted as C [16, 21].

3. Results and Discussion

3.1. NR0 subfamily

The human NR0 subfamily has two members namely, DAX-1 and SHP. A common feature of both these receptors is that they lack a DBD and are orphan receptors.

The sub-cellular localization of DAX1 (Dosage-sensitive sex reversal, Adrenal hyperplasia critical region, on chromosome X, NR0B1) has been reported to be in both nucleus and cytoplasm with slightly higher levels in the nucleus [22]. This information was obtained in experiment on HEK-293 cells using co-immunoprecipitation for the identification of the sub-cellular localization of the protein. The prediction obtained from ngLOC was both nuclear and cytoplasmic localization whereas Hum-mPLoc 3.0 suggested exclusively nuclear localization. The CS obtained for DAX-1 were nuclear (36.32) and cytoplasmic (35.01). The MLCS obtained was 70.4 which confirmed that the receptor exhibits multiple localizations. Thus, considering data from the existing experimental work and our prediction methods, it can be concluded that DAX-1 is primarily distributed between the nuclear and cytoplasmic compartments of the mammalian cell and this distribution may also depend on cell type and physiological conditions.

Previous studies have reported that SHP (Short Heterodimer Partner, NR0B2), localized predominantly in the nucleus [22, 23] using HEK 293 cells as a model. Johannson et al., (1999) [23] used GFP-tagged receptor expression method to obtain the sub-cellular localization of SHP whereas Iyer et al., 2006 [22] utilized Co-IP analysis. The predictions made by both the prediction methods in our present study suggest the localization of SHP to be nuclear which is in accordance with that given in the literature. The CS given for nucleus was 49.91 and the MLCS was 34.86 by ngLOC method. Thus, considering the existing literature and our prediction data, it can be concluded that the unliganded SHP may be localized primarily in the nuclear (N>C) compartment of the cell.

3.2. NR1 subfamily

The NR1 has many members in it and is divided into further seven sub-subfamilies in order to categorize them appropriately such as NR1A, NR1B, NR1C, NR1D, NR1F, NR1H and NR1I. It consists of representative as well as orphan receptors.

Both, ngLOC and Hum-mPLoc 3.0 predicted the sub-cellular localization of TRα (Thyroid hormone Receptor-α, NR1A1) to be nuclear. The CS obtained for nuclear localization was 67.37 whereas the MLCS was found to be 41.87. Previously, reports suggested that, TRα localized primarily in the nucleus of the cell [24] with the help of experiments performed in CV-1 cells using GFP-tagged TRα. Thus, our predictions along with reports from the literature confirmed the localization of the unliganded TRα to be in the nucleus.

Both ngLOC and Hum-mPLoc 3.0 predicted the receptor TRβ (Thyroid hormone Receptor-β, NR1A2) to be nuclear. The CS scores for TRβ were 72.92 (nuclear), 8.456 (cytoplasm) and 2.48 (plasma membrane). The MLCS was given as 28.92 which mean that the receptor is exclusively localized in the nucleus. Previous studies performed in CV1 cells using GFP-tagged TRβ showed that this receptor is predominantly localized in the nucleus (and sparsely in the cytoplasm) [25]. Our predictions are also in-accordance with the reports obtained from

the literature. Thus, from the existing literature and our prediction methods it can be concluded that the localization of unliganded TRβ is predominantly in the nucleus of the mammalian cell.

For RARα (Retinoic Acid Receptor-α, NR1B1), the CS for nucleus and cytoplasm were 44.47 and 34.85 respectively. The MLCS obtained was 71.69, which is above the threshold score set at 60. This indicated (based on ngLOC score) the multiple localization of the receptor (in the nucleus and cytoplasm) in the cells. Hum-mPLoc 3.0 predicted receptor to be localized in the nuclear compartment of the cell. Richter et al., 2002 [26] using normal human prostate epithelium and immune-histochemical localization method for the study of sub-cellular localization of RARα reported its uniform distribution throughout the nucleus and the cytoplasm. Thus, the reports obtained from the literature and the predictions made by both the prediction methods state that the unliganded RARα is localized and distributed both in the nucleus as well as in the cytoplasm.

For RARβ (Retinoic Acid Receptor-β, NR1B2) the results obtained from the ngLOC prediction score suggests the receptor to be localized in both, the nucleus as well as the cytoplasm whereas Hum-mPLoc 3.0 predicted receptor to be localized in the nucleus. The CS obtained were; 49.31 (nucleus), 31.1 (cytoplasm), 3.618 (plasma membrane). The MLCS was given as 65.77. Richter et al., 2002 [26] used the same approach as in case of RARα, to find the localization pattern of RARβ and reported it to be localized in both the nucleus and in the cytoplasm. Thus, from the published literature and our prediction data, the localization of the unliganded RARβ has thus been concluded to be distributed both in the nucleus and cytoplasm.

RARγ (Retinoic Acid Receptor-γ, NR1B3) is reported to be localized in the nucleus as well as in the cytoplasm [26, 27]. Richter et al. (2002) [26] proceeded in same manner as mentioned above in case of RARα and RARβ in order to examine the localization pattern of RARγ while, Hedblom et al., 2013 [27] performed their experiments on U-937 cells using immunofluorescence staining for the same. Both the prediction methods *i.e.* ngLOC [CS- 52.67 (nucleus); MLCS- 51.89] and Hum-mPLoc 3.0 showed the same results *i.e.* localization only in the nucleus. Considering the literature as well as the results obtained from the prediction methods we can conclude that unliganded RARγ may be localized both in the nucleus as well as in the cytoplasm.

PPARα (Peroxisome Proliferator-Activated Receptor α, NR1C1) is reported to be localized in the nucleus of the cell [28]. They performed immunofluorescence assay in COS-7 cell line by transfecting EYFP-tagged (enhanced yellow fluorescent protein) PPARα to obtain its subcellular localization. The predictions made by both the ngLOC and Hum-mPLoc 3.0 suggested that PPARα is localized in the nucleus [CS- 66.86 (nucleus); MLCS- 29.08]. Considering the predicted localization as well as the experimental reports obtained from the literature, the localization of unliganded PPARα is concluded to be nuclear.

PPARβ/δ (Peroxisome Proliferator-Activated Receptor-β/δ, NR1C2) has been previously reported to be localized in the nucleus [28]. Wounded skin section was prepared and then immunofluorescence was performed by immunolabeling against PPARβ using fluorescein isothiocyanate coupled secondary antibody. Both the prediction methods predicted the receptor to be localised in the nucleus. ngLOC predicted with the CS which allotted 62.04 to the nucleus and an MLCS of 31.68. Data availed from the experimental reports and prediction methods indicated that the unliganded PPARβ/δ is localized in the nuclear compartment.

PPARγ (Peroxisome Proliferator-Activated Receptor-γ, NR1C3) has been reported to be localized in the nucleus [29] as well as in the cytoplasm [30, 31]. Berger et al. (2000) [29] used immunofluorescence labeling of transfected COS1 cells and visualized it using a fluorescence microscope to detect the sub-cellular localization of PPARγ. Burgermeister and Seger (2007) [30] reported that HT-29 colon adenocarcinoma cells and human colon epithelial cells display cytoplasmic localization of PPARγ. Moreover, Park et al. (2015) [31] conducted experiments to determine the sub-cellular localization of PPARγ in the prostate tissue of Korean population. Both, ngLOC and Hum-mPLoc 3.0 predicted the protein to be localized in the nucleus. The CS was 74.85 (nucleus) and MLCS was 23.99. The prediction methods predicted the localization to be nuclear and the results, when compared with the report of Berger et al., 2000 [29], are in conclusive agreement. But, contradictory reports of cytoplasmic localization indicated by the other two reports have rendered determination of the localization of the unliganded PPARγ to be in both nucleus and cytoplasm depending on cell type.

Rev-erbAα (NR1D1) has been reported to be localized predominantly in the nucleus [32, 33]. Delannoy et al., 2003 [32] used COS-1 cells and the receptor localization was confirmed by immunofluorescence analysis. Valnegri et al., 2011 [33] used COS-7 cell line and used myc-tagged rev-erbAα to visualize the localization under a fluorescence microscope. Both, ngLOC and Hum-mPLoc 3.0 predicted the protein to be localized in the nucleus. A CS of 23.6 (nucleus) was assigned by ngLOC to the receptor and the MLCS was 34.27. Both reported and predicted localization corroborate with each other thereby concluding the localization of unliganded Rev-erbAα to be in the nucleus.

Rev-erbAβ (NR1D2) has been known to be localized in the nucleus by immunofluorescence assay in fixed COS-1 cells [32]. The predictions obtained from ngLOC as well as Hum-mPLoc 3.0 assigned nucleus as the sub-cellular compartment for the localization of Rev-erbAβ. The CS assigned by ngLOC was 23.86 (nucleus) and the MLCS was observed to be 33.25. The observations made through the prediction methods were validated through experimental reports. Thus, the sub-cellular localization of the unliganded Rev-erbAβ is confirmed to be in the nucleus.

RORα (RAR-Related Orphan Receptor-α, NR1F1) was reported to be localized in the nucleus of the cell [3] using EGFP-tagged RORα in HeLa, HEK 293, MCF-7 and SH-SY5Y cell lines. The ngLOC, as well as Hum-mPLoc 3.0 predictions are similar to the published experimental reports and both the prediction methods predicted RORα to be localized in the nucleus. The CS was 48.09 and the MLCS was 36.13. Thus, comparing the data from the previous experimental work and our prediction methods, it can be concluded that the sub-cellular localization of the unliganded RORα is in the nucleus.

RORβ (RAR-Related Orphan Receptor-β, NR1F2) is reported to be localized in the nucleus (UniProtKB Q92753). Both the prediction methods *i.e.* ngLOC and Hum-mPLoc 3.0 suggested that the receptor is localized in the nucleus. The ngLOC provided with a CS of 31.36 (nucleus) and an MLCS of 36.09. Thus, considering the data primarily derived from the prediction tools, the most probable sub-cellular localization of the RORβ can be concluded to be in the nucleus.

RORγ (RAR-related orphan receptor-γ, NR1F3) is reported to be localized in the nuclear as well as the cytosolic compartments [34]. Normal human skin cells (keratinocytes) were cultured and immunostaining was performed in order to obtain the sub-cellular localization of RORγ. The prediction of sub-cellular localization from both the methods was obtained as nuclear. ngLOC allotted a CS of 28.19 (nucleus) and an MLCS of 36.03. Since, the CS score

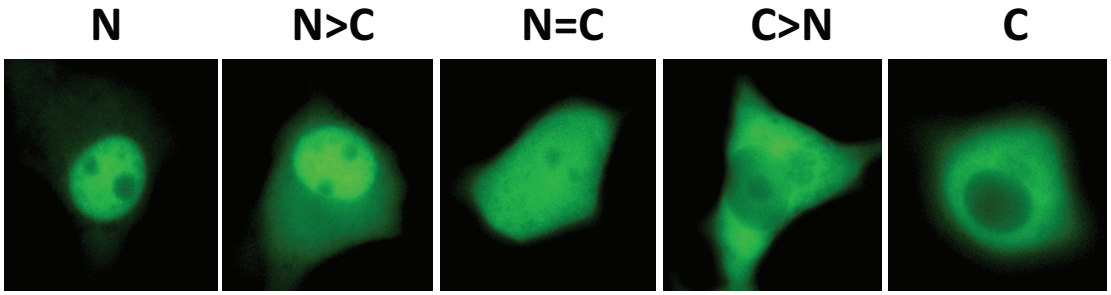

Figure 2: Representative images exhibiting differential sub-cellular localization patterns of a GFP-tagged nuclear receptor in living cells. N= nuclear localization; C=cytoplasmic localization.

for nucleus is too low, a cytoplasmic localization cannot be denied. From the experimental data and our prediction methods, the sub-cellular localization of the unliganded RORγ is assessed to be both nuclear and cytoplasmic.

LXRα (Liver X Receptor-α, NR1H3) has been reported to be localized in the nucleus [35, 36]. Prüfer and Boudreaux, 2007 [35] used YFP-tagged LXRα in HEK-293 cells to obtain the sub-cellular localization of the receptor whereas immunoprecipitation assay was performed by Sakurabashi et al., (2015) [36] using HepG2 cell line to determine the sub-cellular localization of the receptor. The sub-cellular location predicted by both the prediction methods *i.e.* ngLOC and Hum-mPLoc 3.0 were same *i.e.* in the nucleus. The CS given by ngLOC for the receptor was 29.97 (nucleus) and MLCS was 38.25. From the data obtained from the previous reports and our prediction methods, the localization of the unliganded LXRα can be concluded to be in the nucleus.

LXRβ (Liver X Receptor-β, NR1H2) is reported to be localized in the nucleus [35] which is same as of LXRα. The sub-cellular localization of the receptor protein was predicted as nuclear by both ngLOC and Hum-mPLoc 3.0. The CS for the receptor protein was 24.34 (nucleus) and MLCS was 36.12. The predictions, verified through the experimental literature, thus enabled us to conclude that the unliganded LXRβ localizes primarily in the nuclear compartment.

FXR (Farnesoid X Receptor, NR1H4) was reported to be localized in the nucleus by Balasubramaniyan et al., 2013 [37] and Vaquero et al., 2013 [38]. Balasubramaniyan et al., 2013 [37] used HepG2 cells transfected with GFP-tagged FXR and performed immunocytochemistry and immunofluorescence assay whereas Vaquero et al., 2013 [38] used Alexander cells (hepatoma cells), HepG2 (hepatoblastoma), LS-174T (human colorectal adenocarcinoma), HEK-293 cells, and MUM-2C (melanoma) to elucidate the sub-cellular localization of FXR. The predicted sub-cellular localization by ngLOC as well as Hum-mPLoc 3.0 was the nucleus. The CS obtained was 31.17 (nucleus) and the MLCS obtained was 35.15. Literature reports as well as predictions made by prediction methods stated that the unliganded FXR localizes in the nucleus.

VDR (Vitamin D Receptor, NR1I1) has been reported in the past to be multi-localized in the nucleus and the cytoplasm by Gocek et al., 2007 [39] by the use of immunostaining method in THP-1 cells. The predictions made by both the prediction methods exhibited nuclear residency. The CS given by ngLOC was 58.88 (nucleus) and the MLCS was 32.89. Both the prediction methods predicted nuclear localization and the experimental reports also states that the receptor localizes in the nucleus as well as in the cytoplasm. Hence, the localization of unliganded VDR is concluded to be distributed between the nucleus and the cytoplasm.

PXR (Pregnane and Xenobiotic Receptor, NR1I2) was reported to be localized predominantly in the nucleus of the mammalian cells using GFP-tagged PXR and live cell imaging as well as indirect immunodetection in COS-1 as well as HepG2 cells [14]. The predictions given by both the prediction methods indicated nuclear localization of the receptor. The CS obtained from ngLOC was 53.7 (nucleus) and the MLCS obtained was 33.59. Thus, the localization of unliganded PXR is confirmed to be predominantly nuclear.

CAR (Constitutive Androstane Receptor, NR1I3) has been reported to be localized predominantly in the cytoplasm with comparatively lower levels in the nucleus [12, 40] by using GFP- and RFP- tagged receptor in HEK-293T cell lines. The prediction methods (*i.e.* both ngLOC and Hum-mPLoc 3.0) predicted the receptor to be localized in the nucleus alone. The CS obtained from ngLOC was 26.49 (nucleus) and the MLCS was 35.68. Since the CS score was too low, there may be possibility of even cytoplasmic localization. Therefore, through the results obtained from the prediction methods along with the data retrieved from the experimental literature, it can be concluded that the unliganded CAR localizes both in the nucleus and cytoplasm of mammalian cells.

3.3. NR2 subfamily

HNF4α (Hepatocyte Nuclear Factor-4-α, NR2A1) was reported by Jiang et al., 2003 [41] to be localized in the nucleus of the cells by immune-histochemical detection methods using human tissues from the liver, proximal tubules of the kidney and mucosal epithelial cells. The prediction methods predicted the receptor to be localized in the nucleus. The CS obtained from ngLOC was 63.47 (nucleus) and the MLCS was 30.86. The prediction made by the prediction methods is in corroboration with the literature reports and therefore the sub-cellular localization of unliganded HNF4α is confirmed to be nuclear.

HNF4γ (Hepatocyte Nuclear Factor-4-γ, NR2A3) is known to be localized in the nucleus (Uni ProtKB Q14541). Using PROSITE-ProRule annotation, the UniProtKB provided the sub-cellular localization of the receptor to be in the nucleus. The prediction methods also predicted the receptor to be localized in the nucleus. The CS obtained from ngLOC was 52.5 (nucleus) and the MLCS was 33.85. Thus, the localization of this unliganded HNF4γ is concluded to be in the nucleus.

RXRα (Retinoid X Receptor-α, NR2B1) has been reported to be localized in the nucleus [42, 43]. Tanaka et al., 2014 [43] described the sub-cellular localization of the receptor using HMEC (human mammary epithelial cells), MCF-7 and MDA-MB-231 cells by immunofluorescence assays. Karlsson et al., 2004 [42] used normal human epidermal cells to perform immunofluorescence. The prediction methods (both ngLOC and Hum-mPLoc 3.0) predicted the receptor to be localized in the nucleus with a CS of 68.21 (nucleus) and MLCS of 28 given by ngLOC. From the existing literature and our prediction data the sub-cellular localization of unliganded RXRα is concluded to be nuclear.

RXRβ (Retinoid X Receptor-β, NR2B2) was reported by Takiyama et al., 2004 [44] to be localized in the nucleus. Thyroid carcinomas and normal thyroid tissues were subjected to immunohistochemistry in order to find the sub-cellular localization of the receptor. Both the prediction methods showed the receptor to be localized in the nucleus with ngLOC giving a CS of 55.45 (nucleus) and MLCS of 34.66. The localization of this unliganded RXRβ is concluded

to be nuclear after considering results obtained from the prediction methods and reports from the experimental study.

RXRγ (Retinoid X Receptor-γ, NR2B3) is reported to be localized in the nucleus [45]. The experimental method used endometrial epithelial cells. Confocal laser scanning microscopy and immune-electron microscopy were performed to visualize the sub-cellular localization of RXRγ. Both the prediction methods exhibited the receptor to be localized in the nucleus; CS given by ngLOC was 61.23 (nucleus) and the MLCS was 32.14. The predictions along with the literature reports confirmed that the localization of unliganded RXRγ is in the nuclear compartment.

From the existing literature, TR2 (Testicular Receptor 2, NR2C1) is known to be localized in the nucleus (UniProtKB P13056). The data related to the sub-cellular localization of the receptor was given by PROSITE-ProRule annotation method. Both the prediction methods provided similar predictions which predicted the receptor to be localized in the nucleus. The CS obtained from ngLOC was 46.48 and the MLCS obtained was 39.03. The predictions made by both the prediction methods were verified through literature and the unliganded TR2 localization is eventually concluded to be nuclear.

TR4 (Testicular Receptor 4, NR2C2) has been reported to be localized in the nuclear membrane [46] and the nucleus [47]. Yang et al., 2003 [46] used H1299 cells to detect the sub-cellular localization of TR4 by immune-cyto-fluorescence assay. COS-1 cells were used by Nakajima et al., 2004 [47] and immunofluorescence was performed using Flag-CMV-TAK1 and anti-FLAG M2 monoclonal antibody. The predictions obtained from both the methods (ngLOC and Hum-mPLoc 3.0) indicated its nuclear localization with ngLOC CS of 51.56 (nucleus) and MLCS of 37.91. The fact that the prediction methods predicted the localization to be nuclear and that the literature also specifies nuclear localization, the localization of unliganded TR4 is confirmed to be nuclear.

TLX (Homologue of the Drosophila tailless gene, NR2E1) is reported to be localized in the nucleus [48]. Double immunostaining of neural stem cells was performed by Sun et al. (2007) [48] to observe the localization of the TLX receptor. The sub-cellular localization of the receptor was predicted by both the prediction methods as nuclear. The CS obtained from ngLOC was 23.5 (nucleus) and MLCS was 30.3. The prediction methods along with the experimantal reports conclusively state that the localization of unliganded TLX is in the nuclear compartment.

PNR (Photoreceptor cell-specific Nuclear Receptor, NR2E3) is known to be localized in the nucleus of the cell [49]. They used COS-1 cells and immunocytochemistry method to obtain the sub-cellular localization of the receptor. Both the prediction methods show the sub-cellular localization of the receptor to be nuclear. The CS was 52.89 (nucleus) and the MLCS obtained was 33.65. The predictions made by the prediction methods as well as the localization data obtained from the literature are used to state that the sub-cellular localization of the unliganded PNR is in the nucleus.

COUP-TF1 (Chicken Ovalbumin Upstream Promoter Transcription Factor 1, NR2F1) has been previously reported to be localized in the nucleus [50]. The cells used here were from a sea urchin (*Strongylocentrotus purpuratuscells*). Human COUP-TF1 was myc-tagged and was injected into the sea urchin embryo cells by RNA injection of fertilized eggs for observing the sub-cellular localization pattern. Both ngLOC and Hum-mPLoc 3.0 predicted the receptor to be localized in the nucleus of the cell. The CS obtained from ngLOC was 62.14 (nucleus) and the

MLCS obtained was 30.67. Thus, considering data from the existing literature and prediction data, the localization of unliganded COUP-TF1 is concluded to be in the nucleus of the cell.

COUP-TF2 (Chicken Ovalbumin Upstream Promoter Transcription Factor 2, NR2F2) has been reported to be localized in the nucleus by Song et al., 2012 [51] using PPC-1 cell line and GFP-tagged COUP-TF2. The predictions made by the prediction methods ngLOC and Hum-mPLoc 3.0 were nuclear. The CS obtained from ngLOC was 64.91 (nucleus) and the MLCS was 29.26. Considering results obtained from our prediction methods as well as the experimental reports, the sub-cellular localization of the unliganded COUP-TF2 is concluded to be in the nucleus.

EAR-2 (V-erbA-related, NR2F6) has been previously reported to be localized in the nucleus [52]. Jurkat E6-1 cells transfected with GFP-tagged EAR-2 were mixed with Raji B cells and immunofluorescence was performed to observe the sub-cellular localization. Both ngLOC and Hum-mPLoc 3.0 indicated the sub-cellular localization of the receptor to be in the nucleus. The CS obtained from ngLOC was 56.47 (nucleus) and the MLCS was 33.63. The conclusion obtained from both, the experimental as well as the prediction methods, are that unliganded EAR-2 is localized predominantly in the nucleus.

3.4. NR3 subfamily

From the existing literature it has been reported that ERα (Estrogen Receptor-α, NR3A1) is localized predominantly in the nucleus of the cell [53]. Immunofluorescence method was performed in SaOS-2 and HepG2 cells to determine the sub-cellular localization of the receptor. Both ngLOC and Hum-mPLoc 3.0 predicted the receptor to be localized in the nucleus. The ngLOC prediction gave CS 52.21 (nucleus) and the MLCS 57.61. Experimental verification of the localization was carried out in COS-1 cells using GFP-tagged ERα which indicated that the ERα is localized predominantly in the nucleus [54]. The localization of the unliganded ERα is therefore concluded to be nuclear after close examination of the predictions and experimental data.

ERβ (Estrogen Receptor-β, NR3A2) is reported to be localized in the nucleus and cytoplasm of the cell [53]. The cell lines used to report the sub-cellular localization of ERβ were SaOS-2 and HepG2. Immunofluorescence assays were performed and GFP-tagged ERβ was used to determine its sub-cellular localization. Both ngLOC and Hum-mPLoc 3.0 predicted the receptor to be localized in the nucleus. The CS obtained from ngLOC was 71.48 (nucleus) and the MLCS was 27.74. The predictions, as well as the experimental reports are examined, and the conclusion drawn is that the unliganded ERβ is localized primarily in the nucleus with a significant fraction in the cytoplasm.

ERRα (Estrogen-Related Receptor-α, NR3B1) is reported to be localized in the nucleus of the cell [55, 56]. Tremblay et al., 2008 [55] used GFP-tagged ERRα to observe the sub-cellular localization of the receptor in HeLa cells by immunofluorescence analysis. Similarly, Rossi et al., 2011 [56] used RFP-tagged ERRα to identify its sub-cellular localization in HeLa cells and HEK-293T cells by immunofluorescence analysis. Both the prediction methods used here showed the receptor to be localized in the nucleus which is in accordance with the experimental observations. The CS and MLCS obtained from ngLOC were 44.45 (nucleus) and 36.37 respectively. The predictions obtained are in corroboration with the existing experimental literature

and thus the sub-cellular localization of the unliganded ERRα can be concluded to be in the nucleus.

ERRβ (Estrogen-Related Receptor-β, NR3B2) is experimentally shown to be localized in the nucleus of the cell [57]. Immunolocalization was performed in endolymph-producing cells present in the cochlea and ampulla to observe the sub-cellular localization of the receptor. Both the prediction methods suggest the localization of the receptor to be in the nucleus. The CS obtained from ngLOC was 29.65 (nucleus) and the MLCS obtained was 35.78. Data obtained from the prediction methods as well as from the existing experimental work the sub-cellular localization of the unliganded ERRβ can be concluded to be in the nucleus.

ERRγ (estrogen-related receptor-γ, NR3B3) is reported to be localized in the nucleus of the cell (UniProtKB P62508). The sub-cellular localization of the receptor was reported on the website as manually asserted by the curator. Both the prediction methods predicted the sub-cellular localization of the receptor in the nucleus of the cell. The CS obtained from ngLOC was 31.04 (nucleus) and the MLCS obtained was 35.59. Considering the data obtained primarily from the prediction methods, the sub-cellular localization of ERRγ can be concluded to be in the nucleus.

GR (Glucocorticoid Receptor, NR3C1) has been reported to be localized in the cytoplasm [3, 58]. Cell line 1471.1 was used by Htun et al., 1996 [58] to observe the sub-cellular localization of GFP-tagged GR through a confocal laser scanning microscope. Aschrafi et al., 2006 [3] reported that unliganded GR is present exclusively in the cytoplasm of the cell and translocates to the nucleus after binding to its ligand. Prediction tool ngLOC showed the sub-cellular localization to be in the nucleus as well as the cytoplasm of the cell. The CS obtained from ngLOC stated 37.81 (nucleus) and 35.79 (cytoplasm); the MLCS for the receptor was 72.12. But on the contrary, Hum-mPLoc 3.0 predicted the sub-cellular localization to be in the nucleus. The conclusion drawn after careful perusal of the predictions as well as the experimental reports is that the unliganded GR localizes predominantly in the cytoplasmic compartment.

MR (Mineralocorticoid Receptor, NR3C2) is reported to be localized in the nucleus as well as in the cytoplasm of the cell in the absence of its ligand [3, 59]. CV-1 cells were used by Fejes-Tóth et al., 1998 [59] to determine the sub-cellular localization of GFP-tagged MR and fluorescence microscopy was performed in order to visualize the localization in the cells. Aschrafi et al., 2006 [3] mentioned about the sub-cellular localization of MR in general terms. Both the prediction methods predicted the sub-cellular localization of MR to be in the nucleus. The CS obtained from ngLOC was 24.3 (nucleus) and the MLCS was given as 39.81. Since, the CS was too low, a cytoplasmic localization cannot be ignored. On cross-examing the predictions with the experimental literature, the localization of unliganded MR is concluded to be distributed between the nuclear and cytoplasmic compartments.

PR (Progesterone Receptor, NR3C3) is reported to be localized exclusively in the nucleus of the cell in the presence or absence of its ligands [3, 60]. In the studies conducted by Guiochon et al., 1991 [60], 293 cells, COS-7 cells, L mouse cells and MCF7 cells were used for the conduction of immunofluorescence studies in order to observe the sub-cellular localization of the receptor. Aschrafi et al., 2006 [3] generally stated that PR is localized exclusively in the nucleus of the cell. ngLOC predicted the PR to be localized in the nucleus as well as the cytoplasm of the cell whereas Hum-mPLoc 3.0 predicted it to be localized in the nucleus alone. The prediction software ngLOC provided with 37.66 (nucleus) and 30.95 (cytoplasm) as CS

and a score of 64.01 as the MLCS. Considering the data obtained from the prediction methods as well as from the literature, it is concluded that the localization of the unliganded PR is in the nucleus of the cell.

AR (Androgen Receptor, NR3C4) is reported in the literature to be localized in the cytoplasm [61, 62]. Tyagi et al., 2000 [61] expressed GFP-tagged AR in living cells to observe the sub-cellular localization of the unliganded receptor using fluorescence microscope. This enabled the successful identification of the sub-cellular localization of AR. Saporita et al., 2003 [62] also used GFP-tagged AR transfected PC3 cells to observe its sub-cellular localization. The cells were then visualized using a fluorescence microscope. Both ngLOC and Hum-mPLoc 3.0 predicted the localization of the receptor in the nucleus. The CS obtained using ngLOC was 37.93 (nucleus) and the MLCS was 37.34. Both the prediction methods failed to identify the exact localization of unliganded AR which is widely verified experimentally to be in the cytoplasm [61, 62].

3.5. NR4 subfamily

NGFIB/ Nur77 (Nerve Growth Factor 1B / Growth factor-inducible immediate early gene nur 77, NR4A1) is reported in the literature to be localized in the nucleus of the cell [63]. LNCaP cells were used for this purpose and immunofluorescence analysis was conducted on these cells to obtain the sub-cellular localization of the receptor. Both the prediction methods predicted Nur77 to be localized in the nucleus. The CS obtained from ngLOC was 58.11 (nucleus) and the MLCS obtained was 32.55. The predictions coupled with the experimental reports were used to conclude that the localization of this unliganded NGFIB/ Nur77 is in the nucleus.

NURR1 (Nuclear Receptor Related 1, NR4A2) is localized mainly in the nucleus in accordance with the available experimental reports [64]. HEK-293T cells were transfected with GFP-tagged NURR1 and immunofluorescence analysis was conducted to observe the sub-cellular localization of NURR1. Both, ngLOC as well as Hum-mPLoc 3.0, predicted the sub-cellular localization to be in the nucleus. The CS and MLCS obtained from the ngLOC prediction were 66.59 (nucleus) and 29.37 respectively. Considering the reports from the existing experimental work, along with the predictions obtained from our prediction methods, it can be concluded that the sub-cellular localization of unliganded NURR1 resides in the nucleus.

NOR1 (Neuron-derived Orphan Receptor 1, NR4A3) is reported to be localized in the nucleus of the cell in accordance with the experimental work done by Medunjanin et al., 2015 [65]. Immunostaining of human vascular smooth muscle cells were done in order to observe the sub-cellular localization. Both the prediction methods were able to correctly predict the sub-cellular localization of the receptor in the nucleus of the cell. The CS as obtained from ngLOC was 36.88 (nucleus) and the MLCS was 39.27. The experimental reports and the predictions were assessed, and the localization of the unliganded NOR1 is confirmed to be in the nucleus of the cell.

3.6. NR5 subfamily

SF-1 (Steroidogenic Factor 1, NR5A1) is shown to be primarily localized in the nucleus of the cell as reported by Barbara et al., 2000 [66]. The cells used were obtained from the developing-testis and immunofluorescence analysis was conducted in order to visualize the sub-cellular localization of the receptor. The localization predictions made by both the prediction methods are that the receptor is localized in the nucleus of the cell which is acceptable. The CS and MLCS obtained from ngLOC were 59.98 (nucleus) and 31.69 respectively. The predictions along with the experimental reports are utilized to assess the localization of unliganded SF-1 as nuclear.

LRH-1 (Liver Receptor Homolog-1, NR5A2) has been experimentally reported by Choi et al., 2014 [67] to be localized in the nucleus of the cells. Embryonic stem cells were used for the identification of the sub-cellular localization of the receptor with the help of EGFP-tagged LRH-1. Both the prediction methods accurately predicted the sub-cellular localization of the receptor to be in the nucleus with CS and MLCS obtained from ngLOC prediction as 40.51 (nucleus) and 35.13 respectively. The sub-cellular localization of unliganded LRH-1 has been therefore concluded to be nuclear after assessment of the predictions obtained and the existing experimental evidences.

3.7. NR6 subfamily

GCNF (Germ Cell Nuclear Factor, NR6A1) was experimentally reported by Kavarthapu and Dufau, 2015 [68] to be localized in the nucleus of the cell. Adult mice testis sections were used out of which the spermatids and the spermatocytes were used for the identification of the sub-cellular localization. Immunostaining was performed to visualize the cells for the localization of GCNF. Both the prediction methods also made accurate predictions by predicting the sub-cellular localization of the receptor to be in the nucleus. The CS and MLCS obtained from the ngLOC prediction were 29.04 and 49.84 respectively. The experimental reports from literature along with the predictions made by the prediction methods are utilized to conclude that the unliganded GCNF is localized primarily in the nuclear compartment of the cell.

Overall the summary of results and conclusions of the present study has been tabulated in Table-1 for easy understanding and reference purposes (Table I in Supplementary Material available online at `http://www.agialpress.com/journals/nurr/2018/101324/`).

Table 1: The sub-cellular localization of the members of the human nuclear receptor superfamily. *The predictions made by the prediction methods and the evidences drawn after referring the available experimental observations have been tabulated here.*

S.No.	Name and Abbreviation	Prediction from ngLOC	Prediction from Hum-mPLoc-3.0	Experimental observations	Concluding remarks
1.	Dosage-sensitive sex reversal, adrenal hyperplasia critical region, on chromosome X, gene 1/ DAX1	NUC & CYT	NUC	NUC & CYT	NUC & CYT
2	Small heterodimer partner/ SHP	NUC	NUC	NUC	NUC
3	Thyroid hormone receptor-α/ TRα	NUC	NUC	NUC	NUC

S.No.	Name and Abbreviation	Prediction from ngLOC	Prediction from Hum-mPLoc-3.0	Experimental observations	Concluding remarks
4	Thyroid hormone receptor-β/ TRβ	NUC	NUC	NUC	NUC
5	Retinoic acid receptor-α / RARα	NUC & CYT	NUC	NUC & CYT	NUC & CYT
6.	Retinoic acid receptor-β / RARβ	NUC & CYT	NUC	NUC & CYT	NUC & CYT
7.	Retinoic acid receptor-γ / RARγ	NUC	NUC	NUC & CYT	NUC & CYT
8.	Peroxisome proliferator-activated receptor-α / PPARα	NUC	NUC	NUC	NUC
9.	Peroxisome proliferator-activated receptor-β/δ / PPARβ/δ	NUC	NUC	NUC	NUC
10.	Peroxisome proliferator-activated receptor-γ / PPARγ	NUC	NUC	NUC/CYT	NUC/CYT
11.	Rev-erbAα / Rev-erbAα	NUC	NUC	NUC	NUC
12.	Rev-erbAβ / Rev-erbAβ	NUC	NUC	NUC	NUC
13.	RAR-related orphan receptor-α / RORα	NUC	NUC	NUC	NUC
14.	RAR-related orphan receptor-β / RORβ	NUC	NUC	NUC	NUC
15.	RAR- related orphan receptor-γ /RORγ	NUC	NUC	NUC & CYT	NUC & CYT
16.	Liver X receptor-α / LXRα	NUC	NUC	NUC	NUC
17.	Liver X receptor-β / LXRβ	NUC	NUC	NUC	NUC
18.	Farnesoid X receptor / FXR	NUC	NUC	NUC	NUC
19.	Vitamin D receptor / VDR	NUC	NUC	NUC & CYT	NUC & CYT
20.	Pregnane X receptor / PXR	NUC	NUC	NUC	NUC
21.	Constitutive androstane receptor / CAR	NUC	NUC	NUC & CYT	NUC & CYT
22.	Hepatocyte nuclear factor-4-α / HNF4α	NUC	NUC	NUC	NUC
23.	Hepatocyte nuclear factor-4-β / HNF4β	NUC	NUC	NUC	NUC
24.	Retinoid X receptor-α/ RXRα	NUC	NUC	NUC	NUC
25.	Retinoid X receptor-β/ RXRβ	NUC	NUC	NUC	NUC
26.	Retinoid X receptor-γ / RXRγ	NUC	NUC	NUC	NUC
27.	Testicular receptor 2/ TR2	NUC	NUC	NUC	NUC
28.	Testicular receptor 4/ TR4	NUC	NUC	NUC	NUC
29.	Homologue of Drosophila tailless gene / TLX	NUC	NUC	NUC	NUC
30.	Photoreceptor cell specific nuclear receptor / PNR	NUC	NUC	NUC	NUC

S.No.	Name and Abbreviation	Prediction from ngLOC	Prediction from Hum-mPLoc-3.0	Experimental observations	Concluding remarks
31.	Chicken ovalbumin upstream promoter transcription factor 1/ COUP-TF1	NUC	NUC	NUC	NUC
32.	Chicken ovalbumin upstream promoter transcription factor 2/ COUP-TF2	NUC	NUC	NUC	NUC
33.	V-erbA-related/ EAR-2	NUC	NUC	NUC	NUC
34.	Estrogen receptor-α / ERα	NUC	NUC	NUC	NUC
35.	Estrogen receptor-β / Erβ	NUC	NUC	NUC & CYT	NUC & CYT
36.	Estrogen-related receptor-α/ ERRα	NUC	NUC	NUC	NUC
37.	Estrogen-related receptor-β/ ERRβ	NUC	NUC	NUC	NUC
38.	Estrogen-related receptorγ-/ ERRγ	NUC	NUC	NUC	NUC
39.	Glucocorticoid receptor / GR	NUC & CYT	NUC	CYT	CYT
40.	Mineralocorticoid receptor / MR	NUC	NUC	NUC & CYT	NUC & CYT
41.	Progesterone receptor / PR	NUC & CYT	NUC	NUC	NUC
42.	Androgen receptor / AR	NUC	NUC	CYT	CYT
43.	Nerve Growth Factor 1B/ Growth factor inducible immediate early gene nur 77/ NGFIB/ Nur77	NUC	NUC	NUC	NUC
44.	Nuclear receptor related 1/ NURR1	NUC	NUC	NUC	NUC
45.	Neuron-derived orphan receptor 1/ NOR 1	NUC	NUC	NUC	NUC
46.	Steroidogenic factor 1/ SF1	NUC	NUC	NUC	NUC
47.	Liver receptor homolog-1/ LRH-1	NUC	NUC	NUC	NUC
48.	Germ cell nuclear factor/ GCNF	NUC	NUC	NUC	NUC

4. Conclusions

Between ngLOC and Hum-mPLoc-3.0 prediction methods, ngLOC appears to give more accurate predictions about protein localization when compared to the existing literature. From the present comparative study, we conclude that out of 48 human nuclear receptors, when unliganded or inactive, GR and AR exhibit cytoplasmic localization (C). DAX 1, RARα, RARβ, RARγ, PPARγ, RORγ, VDR, CAR, ERβ and MR are either uniformly distributed between nucleus and cytoplasmic compartments (N=C) or may show cell population in the same sample as predominantly nuclear (N>C) and cytoplasmic (C>N) localization. The ratio of this population may also differ depending on cell type or physiological status of the cell which expressing

the receptor. Other NRs show exclusively nuclear localization (N) in the absence of their ligand or when inactive.

NRs, as ligand-modulated transcription factors, are in a dynamic state and are known to shuttle continuously between nuclear and cytoplasmic compartments [7]. For this reason, establishing their subcellular localization in unliganded or inactive state has mostly been controversial. Nonetheless, with the advent of new tools and technologies it has been now possible to present their sub-cellular localization picture with greater confidence. Receptor localization is expected to help towards better understanding of their functioning, control of most of the physiological processes and their mode of action during different pathophysiological conditions.

Competing Interests

The authors declare no competing interests.

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